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Occurrence, analysis and biosynthesis

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Vitamin D in plants
– occurrence, analysis and biosynthesis

Rie Bak Jäpelt
PhD Thesis
2011

DTU Food
National Food Institute

Vitamin D in plants

Occurrence, analysis and biosynthesis

PhD thesis

Rie Bak Jäpelt

Division of Food Chemistry
National Food Institute
Technical University of Denmark
2011

Preface

This PhD study was conducted from 2008 to 2011 at the Division of Food Chemistry, National Food Institute, Technical University of Denmark. The project was financially supported by Ministry of Food, Agriculture and Fisheries, Directorate for Food, Fisheries and Agri Business (3304-FVFP-07-774-02) and Technical University of Denmark.

I will start by expressing my gratitude to everyone who has helped during my PhD study. In particular, I would like to thank my principal supervisor Senior Scientist Jette Jakobsen. Jette has been an invaluable support both in ups and downs. Thank you for giving me diverse and varying tasks and responsibilities and for believing in me. I would also like to thank my co-supervisor, Head of Division Jørn Smedsgaard, for introducing me to mass spectrometry and for valuable discussions on method development.

I thank current and former colleagues at Division of Food Chemistry and especially the group of Bioactive Compounds for creating a comfortable work atmosphere. Special thanks go to Astrid Kvindebjerg for essential technical assistance, this has been highly appreciated.

The collaboration with PhD student Daniele Silvestro and Professor Poul Erik Jensen at Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen has also been rewarding. The studies on vitamin D_3 in plants could not have been performed without their participation. I would also like to thank Thomas Didion, DLF-TRIFOLIUM for help with the studies on vitamin D_2 in grass.

I have been so fortunate to go abroad during my PhD study and special thanks goes to Professor Bruce Hammock and all the people in his lab at University of California Davis, Department of Entomology for welcoming me and for being a great inspiration.

Huge thanks go to friends and family and especially my parents Annette and Christian for always being there for me. You are also the reason for my great interest in chemistry. Last but certainly not least, I would like to thank Jacob. Thank you for your understanding, patience and most of all love.

Rie Bak Jäpelt,

Mørkhøj, December 2011

Summary

The major function of vitamin D in vertebrates is maintenance and regulation of calcium homeostasis and vitamin D is, therefore, critically important for development of a healthy skeleton. Thus, vitamin D insufficiency increases the risk of osteoporosis, but has also been linked to increased risk of hypertension, autoimmune diseases, diabetes and cancer. There is consequently a growing awareness about vitamin D as a requirement for optimal health. Vitamin D_3 is synthesized in the skin by photochemical conversion of provitamin D_3 (7-dehydrocholesterol) by exposure to sunlight at 290-315 nm. However, the necessary wavelengths are not emitted from October to March in Denmark and no vitamin D_3 is consequently synthesized in the skin during winter. Unfortunately, very few food sources naturally contain vitamin D and the general population as a result fail to meet their vitamin D requirements. As a surprise for many is vitamin D present in several plants. The hypothesis, which is the background for this PhD thesis, is that plants can be a source of vitamin D for humans as well as for animals. The overall aim was to study the occurrence and biosynthesis of vitamin D in plants to test this hypothesis.

Most work on vitamin D in plants has been done with non-selective methods such as bioassays and special emphasis was, therefore, placed on development of analytical methods to study vitamin D, its sterol precursors and hydroxylated metabolites in more details. All developed methods were based on liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) detection, because of the superior selectivity and sensitivity. The developed methods were used in three studies covering various aspects of vitamin D in plants.

The term vitamin D includes both vitamin D_2 and vitamin D_3. The provitamin of vitamin D_2 is ergosterol that also is the predominant sterol in fungi. Small amounts of ergosterol can be found in plants contaminated with fungi and the conversion to vitamin D_2 occurs by UVB-exposure of the plant material during growth. Six varieties of perennial ryegrass (*Lolium perenne* L.) were harvested four times during the season and analyzed for ergosterol and vitamin D_2. The average content of vitamin D_2 was 2 µg/kg, which was maximum 2‰ of the ergosterol content. The content of both vitamin D_2 and ergosterol changed more than a factor of ten during the season and a combination of sun and precipitation was important for the synthesis of vitamin D_2.

The synthesis of vitamin D_3 in plants is unresolved and contradicting results concerning the dependence of light have been presented. Various plants were consequently exposed to UVB-light during growth and analyzed for vitamin D_3. Vitamin D_3 was identified in the leaves of *Solanum glaucophyllum* Desf.*, Solanum lycopersicum* L. and *Capsicum annuum* L.*,* belonging to the taxonomic family *Solanaceae*. Vitamin D_3 was found in both UVB- and non-UVB-treated plants, but the content of the UVB-treated plants was 18-64 times higher. No vitamin D_3 was found in *Pisum sativum* L. and *Sorghum bicolor* (L.) Moench belonging to *Fabaceae* and *Poaceae*, respectively. It still needs to be fully established how vitamin D_3 is formed in plants, but both cholesterol and 7-dehydrocholesterol were found in all vitamin D_3 synthesizing plants and may serve as precursors of vitamin D_3 in plants.

Vitamin D is biologically inactive and activation involves two hydroxylations. Vitamin D is first hydroxylated in the liver to 25-hydroxyvitamin D and subsequently to 1,25-dihydroxy vitamin D_3 in the kidneys. An enzymatic pathway similar to that in animals may be present in plants, since enzymatic activity involved in formation of $25OHD_3$ and $1,25(OH)_2D_3$ earlier has been identified in *Solanum glaucophyllum.* The hydroxylated metabolite, 25-hydroxy vitamin D_3, was identified in *Solanum lycopersicum*, *Capsicum annuum* and *Solanum glaucophyllum*. The dihydroxylated metabolite, 1,25-dihydroxy vitamin D_3, was only found in *Solanum glaucophyllum*. Enzymatic hydrolysis was used to study the occurrence of glycoside conjugates. These were found exclusively for 1,25-dihydroxy vitamin D_3 in UVB-treated *Solanum glaucophyllum*.

Altogether, this PhD thesis has shown that both vitamin D_2 and vitamin D_3 can be found in plants. The results demonstrate that grass potentially can be a significant source of vitamin D for grazing animals and animals fed on silage and hay. Especially, leaves from the *Solanaceous* family, where potato and tomato belong, contain high amounts of not only vitamin D_3, but also the hydroxylated metabolites of vitamin D_3. The presence of the hydroxylated metabolites is of particular interest because the activity is believed to be 5-10 times that of vitamin D_3. Further studies are needed to determine if also the fruits contain vitamin D_3. These studies may help to determine whether plants have a potential as a new source of vitamin D.

Resumé (in Danish)

D-vitamins vigtigste funktion er regulering af calciumkoncentrationen i kroppen og D-vitamin har derfor stor betydning for dannelsen af sunde knogler. Lav D-vitaminstatus øger således risikoen for knogleskørhed, men er også blevet forbundet med en øget risiko for cancer, hjertekarsygdomme, diabetes og nedsat immunforsvar. Der har derfor været øget fokus på D-vitamin de seneste år. D_3-vitamin dannes når provitamin D_3 (7-dehydrocholesterol) i huden udsættes for sollys i bølgelængden 290-315 nm. I Danmark kommer solstråler i denne bølgelængde ikke gennem ozonlaget fra oktober til marts og der sker derfor ikke nogen D-vitamin syntese i vinterhalvåret. Ved en normal kost er det svært at få den mængde D-vitamin, der bliver anbefalet da kun fisk indeholder store mængder. Som en overraskelse for mange findes D-vitamin naturligt i flere planter. Hypotesen, der er grundlaget for denne PhD-afhandling er derfor at planter også kan være en kilde til D-vitamin. Det overordnede mål var at undersøge forekomsten og syntesen af D-vitamin i planter med det formål at teste denne hypotese.

D-vitamin i planter blev tidligere analyseret vha. *in vitro* og *in vivo* bioassays, der er relativt non-selektive. I denne PhD-afhandling blev der derfor lagt vægt på udvikling af nye kemiske metoder til analyse af D-vitamin, provitamin D og metabolitter af D-vitamin. Alle de udviklede metoder var baseret på højtryksvæskekromatografi kombineret med tandem massespektrometri (LC-MS/MS) pga. disse metoders gode selektivitet og følsomhed. De udviklede metoder blev anvendt i tre studier med henblik på at undersøge forskellige aspekter af D-vitamin i planter.

Der findes forskellige former for D-vitamin, men D_2-vitamin og D_3-vitamin er de to vigtigste. Provitaminet for D_2-vitamin er ergosterol, men ergosterol er også der mest almindelige sterol i svampe og ergosterol findes derfor i planter kontamineret med svampe. Små mængder D_2-vitamin kan dannes i græs ved UVB-belysning af ergosterol. Seks sorter af almindelig rajgræs (*Lolium perenne* L.) blev høstet fire gange i løbet af en sæson og analyseret for ergosterol og D_2-vitamin. Det gennemsnitlige indhold af D_2-vitamin var 2 µg/kg, hvilket højst udgjorde 2‰ af ergosterol indholdet. Indholdet af både D_2-vitamin og ergosterol ændrede sig mere end en faktor 10 i løbet af en sæson og en kombination af både sol og nedbør havde betydning for dannelsen af D_2-vitamin.

Det vides endnu ikke hvorvidt D_3-vitamin syntesen i planter afhænger af lys. Forskellige planter blev derfor udsat for UVB-lys under vækst og efterfølgende analyseret for D_3-vitamin. D_3-vitamin blev identificeret i blade fra *Solanum glaucophyllum* Desf., *Solanum lycopersicum* L. og *Capsicum annuu*m L., der alle tilhører natskyggefamilien. Både UVB- og ikke UVB-behandlede planter indeholdt D_3-vitamin, men indholdet i de UVB-behandlede planter var 18-64 gange højere. Der blev derimod ikke fundet D_3-vitamin i hverken *Pisum sativum* L. eller *Sorghum bicolor* (L.) Moench, der tilhører henholdsvis ærteblomstfamilien og græsfamilien. Man ved stadig ikke, hvordan D_3-vitamin dannes i planter, men både cholesterol og 7-dehydrocholesterol blev fundet i alle de D_3-vitamin syntetiserende planter og fungerer muligvis som precursorer for D_3-vitamin i planter.

D-vitamin er biologisk inaktiv og aktiveringen involverer to hydroxyleringer. D-vitamin hydroxyleres først til 25-hydroxy vitamin D i leveren og dernæst til 1,25-dihydroxy vitamin D i nyrerne. En lignende biosyntesevej findes muligvis i planter, idet enzymatisk aktivitet involveret i dannelsen af 25-hydroxy vitamin D og 1,25-dihydroxy vitamin D tidligere er blevet fundet i *Solanum glaucophyllum*. Den hydroxylerede metabolit, 25-hydroxy vitamin D_3, blev identificeret i blade fra *Solanum lycopersicum*, *Capsicum annuum* og *Solanum glaucophyllum*. Den dihydroxylerede metabolit, 1,25-dihydroxy vitamin D_3, blev kun fundet i *Solanum glaucophyllum*. Enzymatisk hydrolyse blev brugt til at undersøge forekomsten af glykosid konjugater. Disse blev udelukkende fundet for 1,25-dihydroxy vitamin D_3 i UVB-behandlet *Solanum glaucophyllum*.

Alt i alt har denne PhD-afhandling vist, at både D_2- og D_3-vitamin kan dannes i planter. Græs er potentielt en vigtig D-vitaminkilde for græssende dyr og dyr der fodres med ensilage og hø. Særligt blade fra natskyggefamilien, hvor tomat og kartoffel hører hjemme, indeholder store mængder af ikke kun D_3-vitamin, men også af D_3-vitamins hydroxylerede metabolitter. Forekomsten af de hydroxylerede metabolitter er af særlig interesse, da deres aktivitet menes at være 5-10 gange større end aktiviteten af D_3-vitamin. Yderligere undersøgelser er nødvendige, for at finde ud af om også frugten indeholder D_3-vitamin. Disse undersøgelser kan være med til at vurdere planters potentiale som en ny D-vitaminkilde.

List of publications

This PhD thesis is based on the publications listed below.

Paper I: **Rie Bak Jäpelt**, Daniele Silvestro, Jørn Smedsgaard, Poul Erik Jensen, Jette Jakobsen. LC-MS/MS with atmospheric pressure chemical ionisation to study the effect of UV treatment on the formation of vitamin D_3 and sterols in plants. Food Chemistry, 129: 217-225, 2011. Reprinted with permission from Elsevier.

Paper II: **Rie Bak Jäpelt**, Thomas Didion, Jørn Smedsgaard, Jette Jakobsen. Seasonal Variation of Provitamin D_2 and Vitamin D_2 in Perennial Ryegrass (*Lolium perenne* L.). Journal of Agricultural and Food chemistry, 59: 10907-10912: 2011. Reprinted with permission from American Chemical Society.

Paper III: **Rie Bak Jäpelt**, Daniele Silvestro, Jørn Smedsgaard, Poul Erik Jensen, Jette Jakobsen. Identification of vitamin D_3 metabolites and its sterols precursors in plants. Manuscript submitted for publication in Phytochemistry.

Paper IV: **Rie Bak Jäpelt**, Jørn Smedsgaard, Jette Jakobsen. Review: Vitamin D in plants – biosynthesis, occurrence and function. Prepared to be submitted to Phytochemistry.

List of abbreviations

$1,25(OH)_2D$	1,25-dihydroxy vitamin D
25OHD	25-hydroxy vitamin D
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
C. annuum	*Capsicum annuum* L.
CAS	Cycloartenol synthase
DAD	Diode array detector
DPB	Vitamin D binding protein
Dry wt.	Dry weight
ELSD	Evaporative light scattering detector
ESI	Electrospray ionization
FID	Flame ionization detection
Fresh wt.	Fresh weight
GC	Gas chromatography
HPLC	High performance liquid chromatography
LAS	Lanosterol synthase
LC	Liquid chromatography
LOD	Limit of detection
L. perenne	*Lolium perenne* L.
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
P. sativum	*Pisum sativum* L.
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
PTH	Parathyroidea hormone
RIA	Radioimmunoassay
RRA	Radioreceptorassay
S. bicolor	*Sorghum bicolor* L.
S. glaucophyllym	*Solanum glaucophyllum* Desf.
SIM	Single ion monitoring
S. lycopersicum	*Solanum lycopersicum* L.
SMT	Sterol methyl transferase
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TOF	Time of flight
VDR	Vitamin D receptor

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1 Introduction

The major function of vitamin D in vertebrates is maintenance of calcium homeostasis, but vitamin D insufficiency has also been linked to increased risk of hypertension, autoimmune diseases, diabetes and cancer (Holick, 2004;Lappe et al., 2007;Hyppönen et al., 2001;Pittas et al., 2007;Kendrick et al., 2009;Cantorna and Mahon, 2004). There is consequently a growing awareness about vitamin D as a requirement for optimal health. Vitamin D_3 is synthesized in the skin by photochemical conversion of 7-dehydrocholesterol. However, due to a lack of sunlight during winter, especially in the northern countries, is dietary intake of vitamin D essential. Unfortunately, very few food sources naturally contain vitamin D and the general population as a result fail to meet their vitamin D requirements (Brot et al., 2001;Bailey et al., 2010). Fortification could be part of the solution, but this strategy usually includes very few food items. This makes it difficult to reach all population groups, while toxicity in other groups has to be taken into account and fortification will consequently not meet the vitamin D requirements. A better approach would be to increase the natural food sources of vitamin D.

The hypothesis, which is the background for this PhD thesis, is that plants can be a vitamin D source for humans as well as for animals. The term vitamin D includes both vitamin D_2 and vitamin D_3 and traditionally has only vitamin D_2 been considered to be present in plants. However, very few studies of vitamin D_2 in plants actually exist (Horst et al., 1984;Magalhães et al., 2007). Vitamin D_3 has generally been considered to be absent from plants, but grazing animals in several parts of the world develop calcinosis from consuming specific toxic plants, which is believed to be due to vitamin D_3 (Mello, 2003). Vitamin D_3 or a metabolite of vitamin D_3 present in the plants stimulate calcium absorption producing hypercalcemia and deposition of calcium in soft tissue including aorta, heart, kidney, intestine and uterus (Mello, 2003). However, it is remarkable that so little is known about the production of vitamin D_3 in plants and any improvement in that area will be of great value. The final endpoint would be production of crop plants with a higher natural amount of vitamin D, but to achieve this we need to obtain a better understanding about vitamin D in plants. Most work on vitamin D in plants has been done with non-selective methods such as bioassays and one reason for the limited research could be limitations in the available analytical methods. Selective and sensitive analytical methods are a prerequisite to study vitamin D, its precursors and

metabolites in more details. Special emphasis was therefore placed on development of methods for analysis of vitamin D related compounds in plant material throughout this thesis.

The primary aim of this thesis was to study the occurrence and biosynthesis of vitamin D in plants, with the scope of testing the hypothesis that plants can be a source of vitamin D for humans as well as for animals. Before this aim could be reached several steps were required. First relevant background material had to be collected. Second analytical methods had to be developed to: 1) quantify vitamin D and its sterol precursors 2) identify hydroxylated metabolites of vitamin D_3 and 3) estimate the level of vitamin D conjugates. Finally, this led to the study of the occurrence of vitamin D_2 and vitamin D_3 in plants. Several studies were conducted, by using the developed analytical methods, to:

1. Study the occurrence and seasonal variation of provitamin D_2 and vitamin D_2 in grass
2. Assess the potential of various plants to produce vitamin D_3 by UVB-exposure
3. Study the occurrence of free and conjugated forms of vitamin D_3 metabolites in plants

The thesis is structured as follows: The background material is presented in Chapter 2 and Chapter 3. Chapter 2 provides a brief background on synthesis, metabolism, biological functions, dietary sources and requirements for vitamin D. Chapter 3 and **Paper IV** reviews the literature about vitamin D_2 and vitamin D_3 in plants. Chapter 4 is a summary of the experimental part of this thesis which is the attached three **Papers I-III**. Chapter 5 is a general discussion about the analytical methods used and the challenges confronted with during method development and includes references to **Paper I** and **Paper III**. Chapter 6 is a general discussion about vitamin D in plants with references to **Papers I, II and III**. Based on the described observations are conclusions drawn in Chapter 7. Perspectives with suggestions for further research are given in Chapter 8.

2 Background – Vitamin D

This section provides essential information on vitamin D. Vitamin D synthesis, metabolism and biological functions, as well as dietary sources and recommended intake of vitamin D are described.

2.1 Vitamin D- Synthesis and activation

The discovery of vitamin D began with the research into rickets, which is a bone disease in children that was widespread during the industrial revolution in Europe and United States (Holick, 2004). Both cod liver oil and UV-exposure were described as a curing agent for rickets in the early twentieth century (Mellanby, 1919;Huldschinsky, 1919). The curing agent from cod liver oil was called vitamin D (McCollum et al., 1922). Today ninety years after its discovery, vitamin D deficiency is considered a worldwide problem (Holick and Chen, 2008; Mithal et al., 2009;Kuchuk et al., 2009). Vitamin D is a group of fat-soluble vitamins, which is mainly represented by cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2).

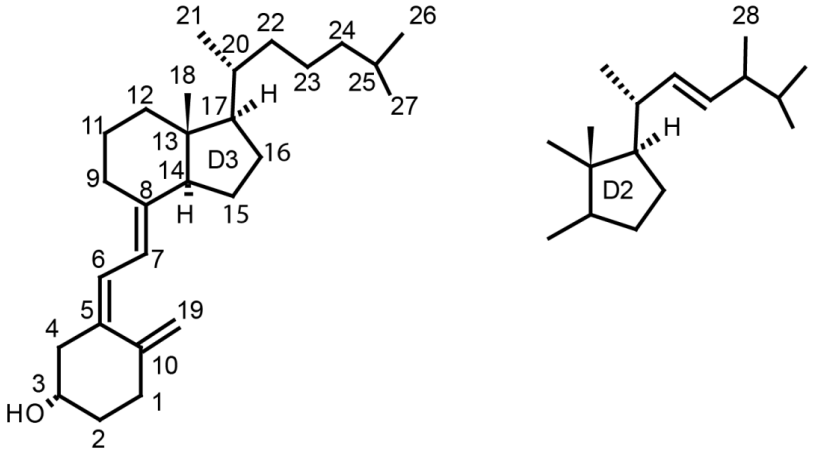

Figure 2.1. Structures of vitamin D_2 ($C_{28}H_{44}O$) and vitamin D_3 ($C_{27}H_{44}O$), the numbering of the carbons are derived from the parent steroid nucleus (modified from Aronov et al., 2008).

Vitamin D_2 and vitamin D_3 differ structurally in the C-17 side chain, which in vitamin D_2 has a double bound and an additional methyl group (Figure 2.1). The vitamins are secosteroids i.e., steroids with a broken ring. In the following vitamin D refers to vitamin D_3 and vitamin D_2, unless otherwise specified. Vitamin D_2 is produced in fungi and yeasts by UVB-exposure of ergosterol (provitamin D_2), whereas vitamin D_3 is produced by UVB-exposure of 7-dehydrocholesterol (provitamin D_3) in the skin (Figure 2.2).

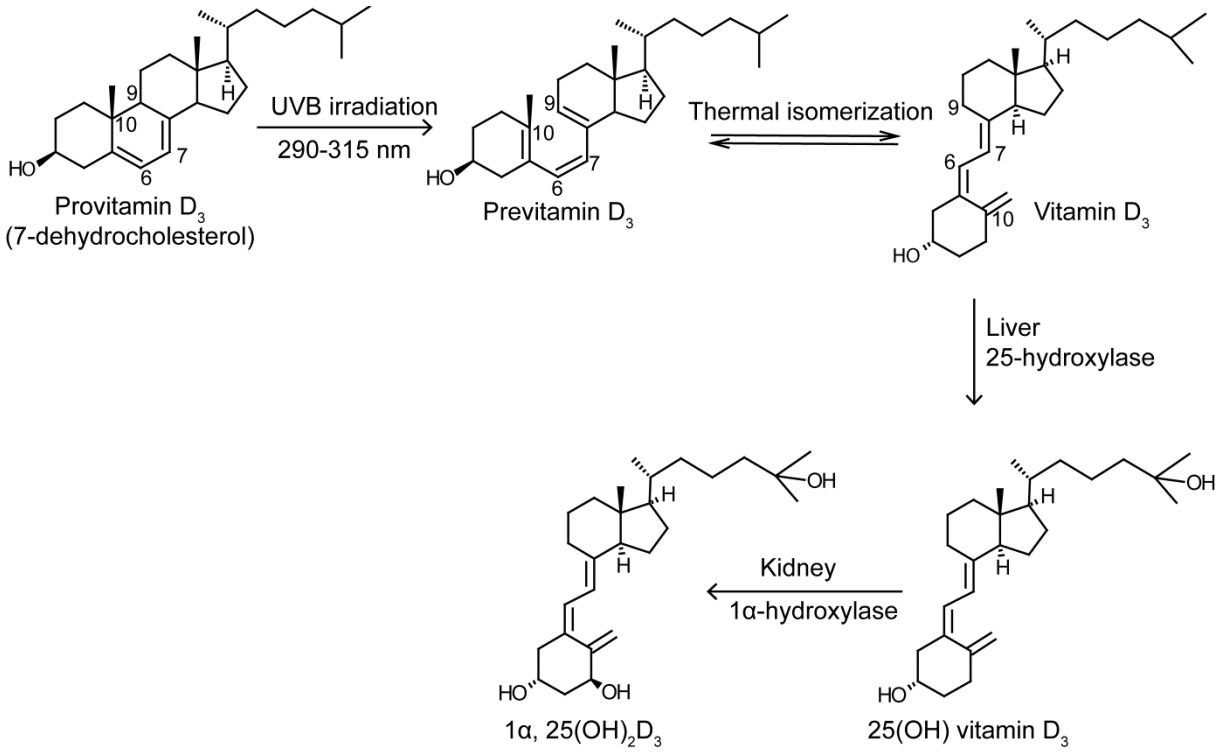

Figure 2.2. Synthesis and activation of vitamin D_3 (modified from Paper III).

The conversion of 7-dehydrocholesterol to previtamin D_3 happens by exposure to sunlight at 290-315 nm (UVB). The optimum wavelengths for the production of previtamin D_3 is between 295 and 300 nm with a maximum near 297 nm (MacLaughlin et al., 1982). Conversion also happens at lower wavelengths, but solar radiation below 290 nm is prevented from reaching the earth's surface by the ozone layer in the stratosphere (MacLaughlin et al., 1982). High-energy photons are absorbed in the conjugated 6,7-diene in the B-ring of ergosterol and 7-dehydrocholesterol which results in ring opening at C-9 and C-10 to form the previtamin D (Havinga, 1973). Previtamin D is biological inactive and thermodynamically unstable and undergoes transformation to vitamin D in a temperature-dependent manner (Havinga, 1973). In vertebrates, the production of vitamin D_3 from 7-dehydrocholesterol, mainly occurs in the two inner layers of the skin (Malpighian layer), the stratum basale and the stratum spinosum (Holick, 1981). Prolonged UVB-radiation converts previtamin D_3 to the inactive forms lumisterol and tachysterol, protecting the organism from vitamin D toxicity (Holick et al., 1981). Synthesis of vitamin D in the skin depends on e.g., season and latitude. The solar zenith angle increases during the winter months and with latitude. Filtration of sunlight through the ozone layer takes place through an increased path length, decreasing the UVB photons that penetrate into the earth's surface. As a result are the rays necessary for

vitamin D production only emitted all year round in places that lie below 35° latitude (Holick, 2003). In the northern hemisphere this is e.g., Northern Africa.

Vitamin D from the skin diffuses into the blood, where it is transported by vitamin D binding protein (DPB) to the liver. Lumisterol and tachysterol have no affinity for DBP and are, therefore, not transported within the blood (Holick, 1981). Ingested vitamin D is absorbed in the small intestine and transported to the liver via chylomicrons and DBP. Vitamin D is biologically inactive and activation involves two hydroxylations (Figure 2.2). Vitamin D is first hydroxylated in the liver at C-25 by a 25-hydroxylase to yield 25-hydroxyvitamin D (25OHD) (reviewed by Prosser and Jones, 2004; reviewed by Jones et al., 1998). The activity of 25-hydroxylase seems to be poorly regulated and dependent primarily on the concentration of vitamin D (Bhattacharyya and DeLuca, 1973). After production of 25OHD in the liver it is transported, bound to DPB, to the kidneys. In passing through the kidneys, 25OHD is hydroxylated at the α-position of C-1 by 1α-hydroxylase to generate 1α,25-dihydroxyvitamin D (1,25$(OH)_2$D) (reviewed by Prosser and Jones, 2004; reviewed by Jones et al., 1998). The bioconversion of 25OHD to 1,25$(OH)_2$D is strictly regulated by serum calcium and serum phosphorus levels, 1,25$(OH)_2$D blood levels and parathyroid hormone (PTH) (reviewed by Prosser and Jones, 2004). Many other tissues do also express 1α-hydroxylase activity, e.g., skin, placenta, colon, brain, osteoclasts, macrophages and the parathyroid glands (reviewed by Brannon et al., 2008).

2.2 Biological effect of vitamin D

Vitamin D has various functions and multiple pathways of action in the body. The active metabolite, 1,25$(OH)_2$D, mediates its biological effect by binding to the vitamin D receptor (VDR). Two pathways exist; a genomic and a non-genomic (Norman et al., 1992). The genomic pathway is mediated by VDR, which on binding to 1,25$(OH)_2$D in the cytosol interacts with DNA to induce or inhibit new protein synthesis (Norman et al., 1992). An example of a genomic pathway is the binding of 1,25$(OH)_2$D in the intestinal cells that allow VDR to act as a transcription factor in the expression of transport proteins involved in transport of calcium (Norman et al., 1992). The non-genomic pathway includes interactions with VDRs in the cell membrane. Rapid intestinal absorption of calcium is one effect mediated by the non-genomic pathway (Norman et al., 1992). The non-genomic pathway is

usually working very fast i.e., within seconds and minutes, whereas genomic responses typically take a few hours to days (Norman, 2006).

The main function of vitamin D is maintenance and regulation of calcium levels in the body. Low blood calcium stimulates release of PTH from the parathyroid gland. PTH stimulates in turn 1α-hydroxylase in the kidneys to produce $1,25(OH)_2D$, which then increases serum calcium concentrations by acting on three targets: increased absorption from the intestine, reabsorption in the kidneys and mobilization from bones (reviewed by DeLuca, 2004). Vitamin D also stimulates renal absorption and active intestinal absorption of phosphorus (reviewed by DeLuca, 2004). Without sufficient vitamin D humans will develop a deficiency disease. Growing children develops rickets because of failure in calcification of cartilaginous growth plates. Osteomalacia develops in adults during prolonged vitamin D deficiency, where the newly formed uncalcified bone tissue gradually replaces the old bone tissue with weakened bones as a consequence.

VDR is present in the enterocytes, osteoblasts and distal renal tubules where vitamin D has its main functions, however VDR has been found in most tissues e.g., parathyroid gland cells, brain, heart, lymphocytes, skin, pancreas, prostate, gonads, breast and mononuclear cells (reviewed by Holick, 2004;reviewed by DeLuca, 2004). The expression of VDR in these cells suggest that they have a function there and $1,25(OH)_2D_3$ has indeed been shown to have a multitude of other physiological functions not related to calcium homeostasis (reviewed by Holick, 2004;reviewed by DeLuca, 2004). This helps explain why vitamin D insufficiency has been associated with such diverse diseases as cancers (Holick, 2004;Lappe et al., 2007), cardiovascular diseases (Kendrick et al., 2009), autoimmune diseases (Cantorna and Mahon, 2004), and diabetes (Hyppönen et al., 2001;Pittas et al., 2007).

2.3 Dietary intake of vitamin D

2.3.1 Sources of vitamin

Because the body produce vitamin D_3, vitamin D does not meet the classical definition of a vitamin. Diet is a secondary source of vitamin D, if sun exposure is adequate, since only few foodstuffs contain significant amounts of vitamin D. However, in wintertime, when the vitamin D needs cannot be met by endogenous production, the dietary intake of vitamin D

becomes essential. Generally, fish have the highest natural amount of vitamin D_3 and the dietary intake of fish is, therefore, essential. Other significant sources of vitamin D_3 are meat, egg and milk products (Table 2.1).

Table 2.1. Selected food sources of vitamin D. All data is taken from the Danish Food Composition Database (Saxholt, E., Christensen, A.T., Møller, A. Hartkopp, H.B., Hess Ygil, K., Hels, O.H., 2009)

Food	µg/100g
Cod liver oil	250
Salmon, raw	30
Mackerel, raw	5.45
Tuna, raw	2.90
Milk, whole (3.5%)	0.100
Milk, skimmed (0.5%)	0.076
Cheese (30%)	0.196
Egg, whole, raw	1.75
Mayonnaise	1.0
Pork, minced, raw	0.54
Beef, mince, raw	0.6

A high intake of meat and milk products is typical for a western diet and the contribution to the total vitamin D intake is consequently significant despite a lower content of vitamin D compared to fish. The content of vitamin D in food of animal origin depends on what the animal has been fed (Mattila et al., 1999;Jakobsen et al., 2007;Graff et al., 2002). The main compound in food is vitamin D_3, but the metabolites which are part of the metabolic pathway in vertebrates may also be present (Mattila et al., 1995b;Mattila et al., 1995a;Clausen et al., 2003;Jakobsen and Saxholt, 2009). Food sources of vitamin D_2 are very limited and wild mushrooms are one of the only significant sources of vitamin D_2 (Mattila et al., 1994;Mattila et al., 2002;Teichmann et al., 2007). Milk from dairy cows contain a significant although low amount of vitamin D_2, which is expected to derive from grass and hay (Jakobsen and Saxholt, 2009).

Vitamin D fortification of selected foods has been accepted as a strategy to improve the vitamin D status of the general population both in United States and in many European countries. Milk and margarine are the primary products that are enriched with vitamin D

(Natri et al., 2006), but also orange juice (Calvo et al., 2004), bread (Natri et al., 2006;Hohman et al., 2011), cheese and yoghurt may be enriched (Holick, 2011). This area is regulated differently in each country and fortification may either be voluntary or mandatory and the levels added vary accordingly. In Denmark, fortification with vitamin D is in general not used and do consequently not contribute significant to the dietary intake. However, the intake of multivitamin supplements is very common in Denmark (Rejnmark et al., 2009).

The determination of vitamin D and its metabolites in food is a challenge, which will be discussed within this thesis. Beyond the scope of this PhD thesis, another challenge is to assess the total vitamin D activity in food to estimate the dietary intake of vitamin D. However, a short introduction to this topic is given. Results from chemical methods need to be corrected for bioavailability and activity of each of the metabolites to calculate the total vitamin D activity. This is difficult since studies investigating the relative activity of vitamin D metabolites are limited. Some studies indicate that vitamin D_2 is less effective than vitamin D_3 in maintaining vitamin D status (Armas et al., 2004;Trang et al., 1998), whereas other question this (Holick et al., 2008;Rapuri et al., 2004). The potency of 25OHD has often been attributed to possess five times the potency of vitamin D (Reeve et al., 1982;Jakobsen, 2007), but recent research in this area question this (Jakobsen et al., 2007;Jakobsen, 2007). The potency of $1,25(OH)_2D$ has been attributed to ten relative to vitamin D (Tanaka et al., 1973), however this value is not implemented in food composition tables, as there is no specific composition data available for the content of $1,25(OH)_2D$.

2.3.2 Dietary recommendations

The Danish dietary vitamin D recommendations are based on the Nordic Nutrition Recommendations (NNR). NNR 2004 recommends 10 µg/day for newborns and small children (6-23 months), elderly (≥61 years), pregnant and breastfeeding and 7.5 µg/day for the age group 2 to 60 years (NNR, 2004). The circulating concentration of 25OHD is the accepted biomarker for vitamin D status, as this reflects both dietary intake and skin production. The optimal vitamin D status has been a subject of debate and there is no general standard for optimal 25OH status (Dawson-Hughes et al., 2005). The Danish National Board of Health defines a plasma concentration below 50 nmol/L as vitamin D insufficiency,

whereas below 25 nmol/L is vitamin D deficiency and below 12.5 nmol/L is severe vitamin D deficiency.

Excessive vitamin D consumption can result in toxicity. Toxic levels are not obtained by an usual diet, but by excessive consumption of vitamin D supplements or over-fortification of food. Vitamin D intoxification is primarily due to hypercalcemia caused by increased intestinal absorption of calcium, together with increased resorption of bone. If the vitamin D exposure is prolonged, deposition of calcium in soft tissues particularly in arterial walls and in the kidney occurs. The tolerable upper intake level for humans is set to 50 µg/day (NNR, 2004).

3 Background – vitamin D in plants

"Vitamin D in plants: Biosynthesis, occurrence and function" is reviewed in ***Paper IV****.* ***Paper IV*** *summarizes the current knowledge on sterol biosynthesis leading to 7-dehydrocholesterol. The quality of previous detection methods used to study vitamin D and related compounds are also discussed in parallel with the content of vitamin D and its hydroxylated metabolites in higher plants and in plankton. A summary of the most important subjects in relation to the scope of this PhD thesis is given in this chapter.*

3.1 Vitamin D_3 in plants

3.1.1 Vitamin D_3 and 7-dehydrocholesterol

Grazing animals in several parts of the world develop calcinosis from consuming specific toxic plants. The hypothesis is that, similar to human toxicity, excess vitamin D stimulates calcium absorption producing hypercalcemia and deposition of calcium in soft tissue including aorta, heart, kidney, intestine and uterus (Mello, 2003). The symptoms are loss of weight, bent forelegs, reduced milk production, increased heart rate and possibly death (Rambeck et al., 1979). Most work has been conducted with the plant *Solanum glaucophyllum* Desf. (*S. glaucophyllum*). Controlled studies with various animals including rabbits (Mautalen, 1972; Dallorso et al., 2008;Humphreys, 1973), chickens (Wasserman et al., 1976a;Weissenberg et al., 1989) and rats (Uribe et al., 1974;Basudde and Humphreys, 1976) verified that *S. glaucophyllum* or an extract caused an increased absorption of calcium and phosphorus. Also *Cestrum diurnum* L. and *Trisetum flavescens* Beauv. have caused calcinotic diseases in horses and cattle (Wasserman et al., 1975). These findings later led to the search for vitamin D_3 in plants by chemical methods. The plant species in which vitamin D_3 and/or 7-dehydrocholesterol have been identified by chemical methods are listed in Table 3.1. The plant species belong to *Solanaceae* (*Solanum lycopersicum* L.*, Solanum tuberosum* L.*, Solanum glaucophyllum* Desf.*, Nicotiana glauca* Graham*,, Cestrum diurnum* L.), *Cucurbitaceae* (*Cucurbita pepo* L.)*, Fabaceae* (*Medicago sativa* L.) and *Poaceae* (*Trisetum flavescens* Beauv.). Although several species have been shown to contain vitamin D_3, most of them belong to the taxonomic family *Solanaceae* (Aburjai et al., 1998;Curino et al., 1998;Prema and Raghuramulu, 1996;Skliar et al., 2000;Esparza et al., 1982;Prema and Raghuramulu, 1994).

Table 3.1. Plants in which vitamin D_3 and 7-dehydrocholesterol either have been quantified or identified by chemical methods (Modified from Paper IV)

Species	Vitamin D_3	7-dehydrocholesterol
Solanum lycopersicum L.	0.28 µg/g dry wt.[1] 1.1 µg/g fresh wt.[2] 0.8 ug/g dry wt.[3]	0.61-0.76 µg/g dry wt.[1]
Solanum tuberosum L.	0.15 µg/g fresh wt.[1]	-
Cucurbita pepo L.	0.23 µg/g fresh wt.[1]	-
Solanum glaucophyllum Desf.	0.21 µg/g dry wt.[2] 2.2-42.1µg/g fresh wt.[4] Identified[5,6]	- 5-58µg/g fresh wt.[4] Identified[5,6]
Nicotiana glauca Graham.	Identified[7]	Identified[7]
Cestrum diurnum L.	0.1 µg/g fresh wt.[8]	-
Medicago sativa L.	0.00062-0.001 µg/g dry wt.[9]	-
Trisetum flavescens Beauv.	0.1 µg/g dry wt.[10]	-

[1]Björn and Wang (2001), [2]Aburjai et al. (1998), [3]Prema and Raghuramulu (1996), [4]Aburjai et al. (1996), [5]Curino et al. (2001), [6]Curino et al. (1998), [7]Skliar et al. (2000), [8]Prema and Raghuramulu (1994), [9]Horst et al. (1984), [10]Rambeck et al. (1979)

Boland et al. (2003) states that it is unexpected that independent traits can take place several times in the evolution of different families and hypothesized that the ability to synthesize vitamin D_3 is a characteristic of angiosperms in general (Review by Boland et al., 2003). However, this remains to be established. Vitamin D_3 appears to be synthesized in plants mainly by a photo-dependent process similar to what occurs in the skin of vertebrates (Zucker et al., 1980;Björn and Wang, 2001;Aburjai et al., 1996), but data supporting the action of a non-photolytic reaction also exists (Curino et al., 1998).

3.1.2 Hydroxylated metabolites of vitamin D_3 in plants

The hydroxylated metabolites of vitamin D_3 have been found in several plants (Table 3.2). An enzymatic pathway similar to that in animals may be present, since enzymatic activity involved in formation of $25OHD_3$ and $1,25(OH)_2D_3$ has been identified in *S. glaucophyllum* (Esparza et al., 1982). Vitamin-D-25-hydroxylase activity was localized in the microsomes, whereas the 1α-hydroxylase activity was localized in mitochondria and microsomes (Esparza et al., 1982). Attempts were later made to demonstrate hydroxylation of vitamin D_3 in *Populus tremula* L. using ^{14}C-labelled vitamin D_3, but without success (Pythoud and Buchala, 1989). This suggests that vitamin D_3 metabolism is different from vitamin D_3-rich plants such as *S. glaucophyllum* (Pythoud and Buchala, 1989).

Table 3.2. Occurrence of hydroxylated metabolites of vitamin D_3 in various plants (Paper IV)

Species	$25OHD_3$	$1\alpha,25(OH)_2D_3$
Solanum lycopersicum L.	0.15 μg/g fresh wt.[1] 0.022 μg/g dry wt.[2]	- 0.10 μg/g dry wt.[2]
Solanum glaucophyllum Desf.	Identified[3,4] 1.0 μg/g fresh wt.[5]	Identified[3,4] 0.1 μg/g fresh wt.[5]
Cestrum diurnum L.	0.102 μg/g dry wt.[6]	1 μg/g dry wt.[6]
Nicotiana glauca Graham	Identified[7]	0.3-1 μg/g fresh wt.[7]

[1]Aburjai et al. (1998), [2] Prema and Raghuramulu (1996), [3]Curino et al. (1998),[4]Esparza et al. (1982), [5]Aburjai et al. (1996), [6]Prema and Raghuramulu (1994), [7]Skliar et al. (2000)

3.1.3 Vitamin D conjugates

Sterols are commonly found in free form in plants, but acetylation and glycosylation happens in varying degree. The sterols are either acetylated with fatty acids or conjugated with sugars, which also can be acetylated. These are generically called sterol conjugates (Figure 3.1). Steryl esters are present in all plants and are most often localized in cytoplasm of plant cells (reviewed by Benveniste, 2002). They do typically represent a storage form of sterols in plants (review by Piironen et al., 2000). Steryl glycosides usually consist of a mixture differing in saccharide moiety and especially plants from the *Solanaceae* family demonstrate an unique abundance of glycosides (Moreau et al., 2002;Potocka and Zimowski, 2008).

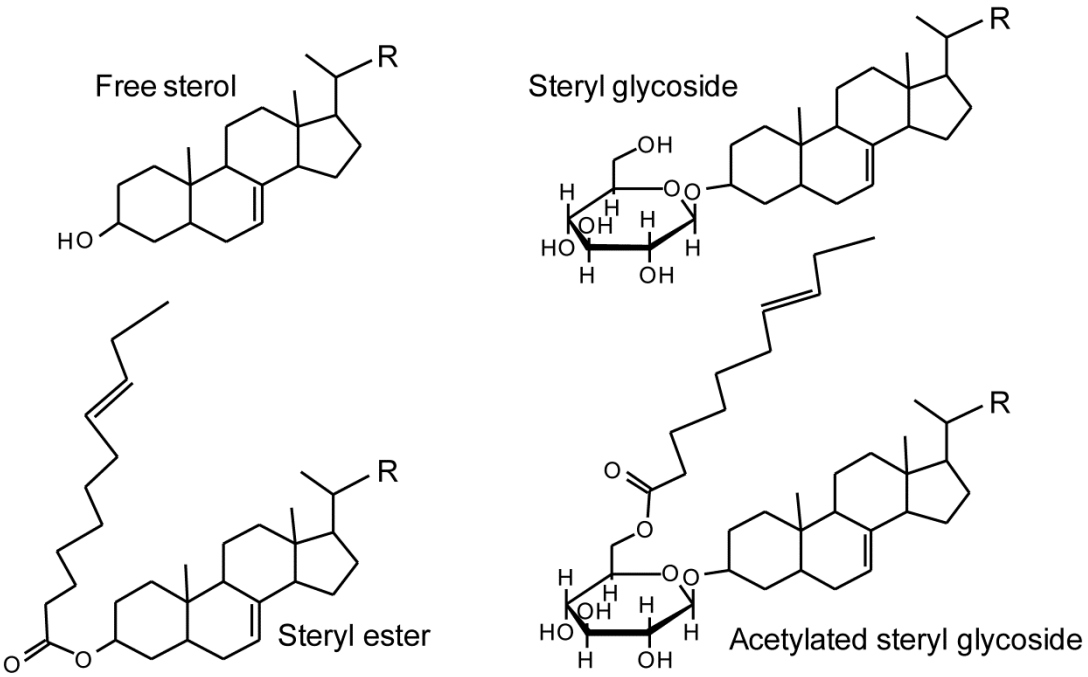

Figure 3.1. Basic structures of sterol conjugates. R side chain varies between sterols (Paper IV).

Early work identified $1,25(OH)_2D_3$ in *S. glaucophyllum* after enzymatic hydrolysis with mixed glycosidases (Esparza et al., 1982;Wasserman et al., 1976b;Napoli et al., 1977;Haussler et al., 1976;Hughes et al., 1977) and in a similar study in *Cestrum diurnum* (Hughes et al., 1977). Vitamin D_3 and $25OHD_3$ have also been identified in *S. glaucophyllum* after incubation with a glycosidase (Esparza et al., 1982). This indicates that vitamin D_3 and its metabolites are bound as glycosides. However, the existence of glycosides is debated and other studies mainly detected the free forms (Aburjai et al., 1998;Prema and Raghuramulu, 1996;Aburjai et al., 1996).

3.2 Biological function of vitamin D_3 metabolites in plants

The biological function of vitamin D_3 and its metabolites in plants remains unclear. The theory is that vitamin D or vitamin D-like substances act as plant growth substances mediated by changes in calcium fluxes. Vitamin D_2 and vitamin D_3 enhance the number of adventitious roots in *Populus tremula* L., *Populus nigras* L. (Buchala and Schmid, 1979), *Phaseolus vulgaris* L. (Buchala and Schmid, 1979;Talmon et al., 1989) and in *Phaseolus aureus* Roxb. (Jarvis and Booth, 1981). Of the metabolites of vitamin D_3, only $1,25(OH)_2D_3$ promotes adventitious rooting, but to a smaller extent than vitamin D_3 (Pythoud et al., 1986). Glycosides of several vitamin D_3 metabolites promote rooting to the same extent as the parent metabolite (Pythoud et al., 1986).

The effect of vitamin D_3 on root growth is suggested to be connected to calcium uptake and synthesis of calcium-binding protein (calmodulin) (Talmon et al., 1989;Vega et al., 1985). Vitamin D_3 has been demonstrated to stimulate calmodulin synthesis and Ca^{2+}-uptake in *Phaseolus vulgaris* root segments *in vitro* (Vega et al., 1985;Vega and Boland, 1986). Vitamin D_3 applied to potato plantlets stimulates Ca^{2+} uptake, but also UV irradiation stimulates Ca^{2+} uptake, which may be a result of photo-dependent vitamin D_3 synthesis (Habib and Donnelly, 2005). Vitamin D has also been demonstrated to stimulate cell division in root meristems (Talmon et al., 1989). It has been suggested that vitamin D_3 stimulates DNA synthesis in the tissue of root meristem at least partly through changes in intracellular calcium and calmodulin (Vega and Boland, 1986;Vega et al., 1989). Milanesi and Boland (2006) identified a protein similar to the vertebrate VDR in *S. glaucophyllum*, whether the VDR-like proteins play a functional role remains to be established.

The 7-dehydrocholesterol/vitamin D_3 system has also been proposed to act as a UVB-sensor (Björn and Wang, 2001). 7-dehydrocholesterol is suitable as a radiation sensor because of the high quantum yield for photoconversion (Björn and Wang, 2001). As mentioned in section 2.1, the action spectrum for conversion of 7-dehydrocholesterol to vitamin D_3 show a peak at 297 nm (MacLaughlin et al., 1982). Since this UV absorption spectrum completely overlaps the UV absorption spectra for DNA, RNA and proteins is it possible that 7-dehydrocholesterol evolved to protect the UVB-sensitive macromolecules from UVB-exposure (Holick, 2003).

3.3 Vitamin D_2 in plants

UVB-exposure of various vegetables and crops in the beginning of the twentieth century yielded antirachitic activity (Hess and Weinstock, 1924), which was stated to be vitamin D_2 produced from ergosterol. Ergosterol is the predominant sterol found in fungi, and small amounts can be found in plants contaminated with fungi. The conversion to vitamin D_2 occurs by UVB-exposure of the plant material during growth and in the curing process. Grass could therefore be an important source of vitamin D for grazing animals and animals fed on silage and hay. Vitamin D in grass and hay was studied intensively 50-80 years back using biological assays (Thomas and Moore, 1951;Thomas, 1952;Wallis, 1939;Wallis, 1938;Wallis et al., 1958;Russell, 1929;Newlander and Riddell, 1952;Newlander, 1948;Henry et al., 1958;

Steenbock et al., 1925;Moore et al., 1948;Keener, 1954). However, very little recent information exists on the content of vitamin D_2 in plants (Horst et al., 1984;Magalhães et al., 2007).

3.4 Vitamin D biosynthesis

Sterols act as precursors of steroids including vitamin D, thus ergosterol is a provitamin for vitamin D_2 and 7-dehydrocholesterol for vitamin D_3. Sterols are made up of a tetracyclic system with a long side chain at C-17 and four rings designated A, B, C and D (Figure 3.2). The sterols can in general be divided in C27, C28 and C29 sterols, where the C28 have a methyl group at C-24 and the C29 an ethyl group at C-24. C27 sterols are typical for vertebrates, whereas the C28 and C29 are typical for plants. Sterols are components of membranes and have a function in regulation of membrane fluidity and permeability. Cholesterol in animals and ergosterol in fungi fulfil this role, whereas the plant kingdom in comparison produces very diverse sterols (Schaeffer et al., 2001). More than 200 sterols have been found in plants, but sitosterol, campesterol and stigmasterol normally predominates (Lagarda et al., 2006).

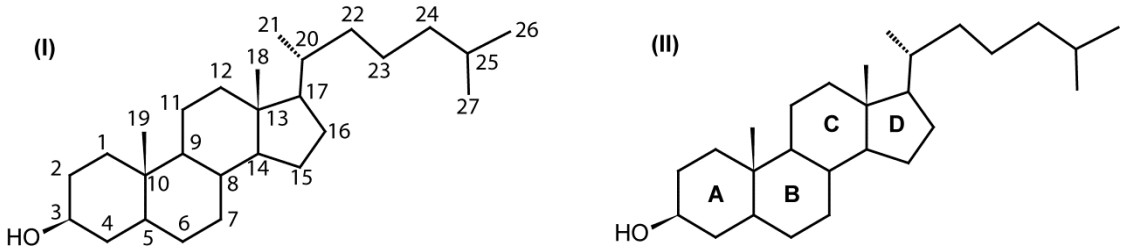

Figure 3.2.Sterol structures (I) with numbering of the carbon atoms and (II) with naming of the rings (Paper IV).

To understand how vitamin D_3 synthesis takes place in plants we need to understand how its sterol precursors are formed. A short review of the key steps of sterol biosynthesis leading to vitamin D_3 is, therefore, needed. The sterols are built through a complex biosynthesis from the common C5 isoprene units isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The intermediate to form these isoprene units are for sterols mevalonic acid (MVA) (Rodrıguez-Concepción and Boronat, 2002). MVA itself is a product of acetate metabolism and three molecules of acetyl-CoA are initially combined to make the branched-chain ester *S*-3-hydroxy-3-methylglutaryl-CoA (*S*-HMG-CoA), which is reduced to MVA. MVA is then phosphorylated to MVA-5-diphosphate in two steps, which is converted

to IPP and later isomerized to DMAPP. One molecule of DMAPP and two molecules of IPP are then assembled into farnesyl diphosphate (C15). Two molecules farnesyl diphosphate are finally combined to make squalene (C30). The process from MVA to squalene is the same for all eukaryotes, but large differences exist downstream from squalene (reviewed by Benveniste, 2002). Cyclization of squalene is via the intermediate 2,3-oxidosqualene, that forms either lanosterol or cycloartenol via a series of enzymatic cyclizations (Figure 3.3). Ergosterol and cholesterol are synthesized via lanosterol catalyzed by lanosterol synthase (LAS), in fungi and in vertebrates, respectively (Ohyama et al., 2009). Plants sterols are synthesized via cycloartenol catalyzed by cycloartenol synthase (CAS) in higher plants (Ohyama et al., 2009).

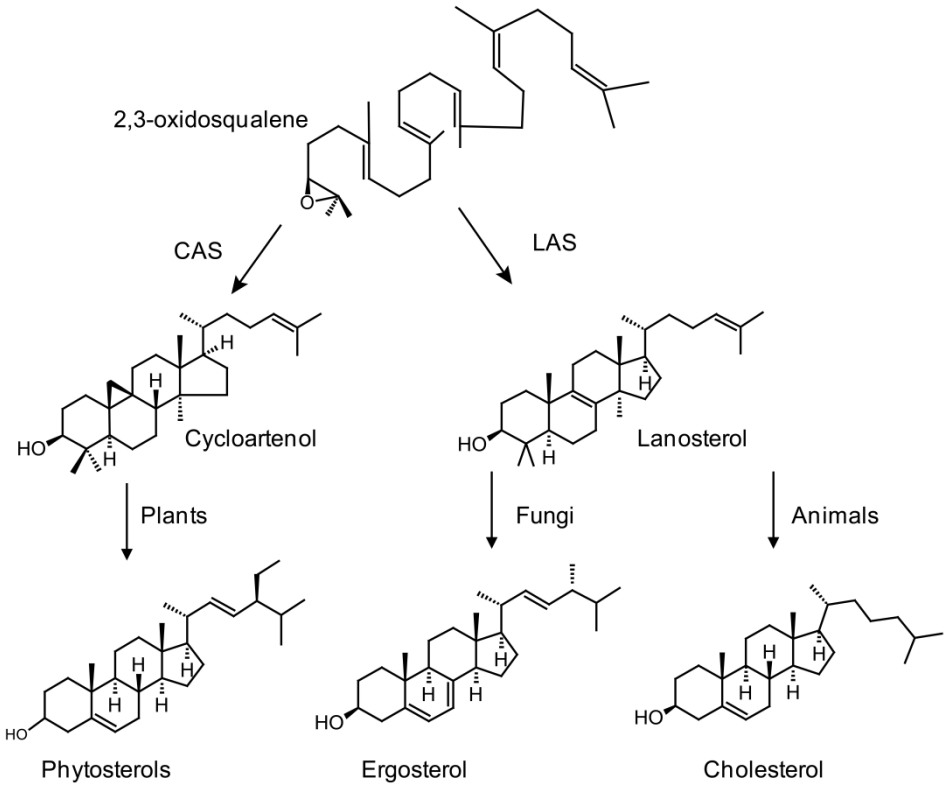

Figure 3.3. Cyclization of 2,3-oxidosqualene that forms either lanosterol or cycloartenol via a series of enzymatic cyclizations leading to sterols in plants, fungi and animals (Paper IV).

3.4.1 Postsqualene sterol biosynthesis in animals and fungi

The simplified cholesterol and ergosterol pathway downstream from lanosterol is shown in Figure 3.4. The conversion of lanosterol to cholesterol involves removal of three methyl groups, reduction of double bonds and migration of the double bond in lanosterol to a new position in cholesterol (Waterham et al., 2001). The biosynthesis from lanosterol to cholesterol is a 19-step process that requires nine different enzymes (reviewed by Risley, 2002) and details will not be given here. The sequence of reactions in the cholesterol

biosynthetic pathway may vary (Waterham et al., 2001). Alternate routes exist because reduction of the C-24,25 double bond on the hydrocarbon side chain of the sterol ring structure by sterol Δ^{24}-reductase can occur at multiple points in the pathway, giving rise to various intermediates (Bae and Paik, 1997). These intermediates, with or without a double bond in the hydrocarbon side chain, can serve as substrates for the other enzymes in the pathway. Ergosterol shares the pathway with cholesterol until zymosterol (Lees et al., 1995, Figure 3.4).

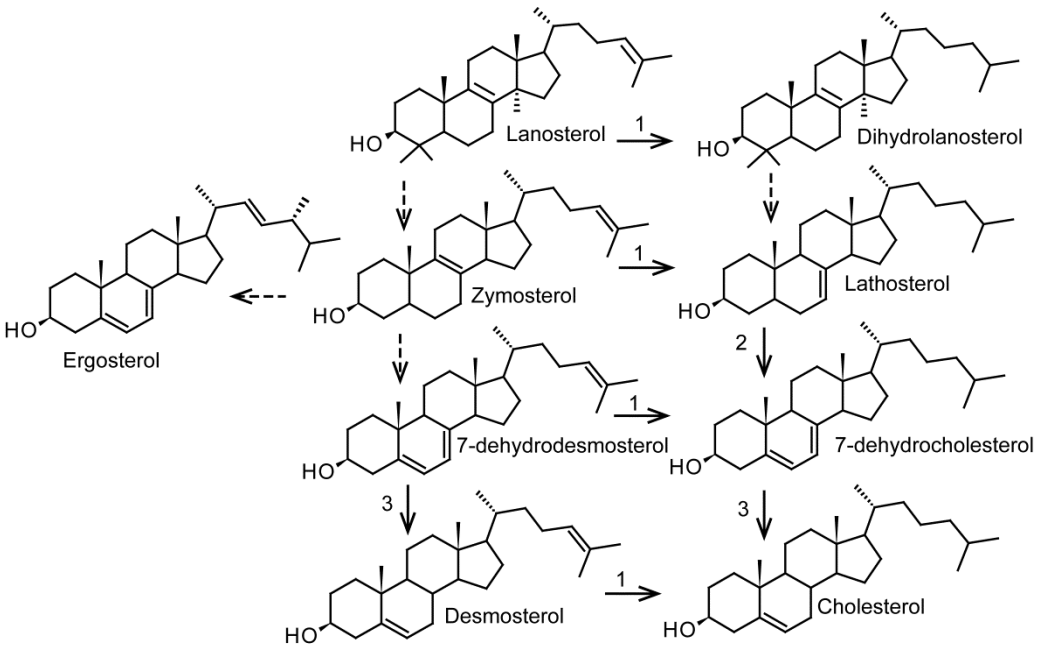

Figure 3.4. Simplified cholesterol and ergosterol biosynthesis pathway. Dashed arrows corresponds to multiple enzymatic reactions. Enzymes are denoted by numbers. 1: sterol-Δ^{24}-reductase, 2: lathosterol 5-desaturase, 3: $\Delta^{5,7}$-sterol-Δ^{7}-reductase (Paper IV).

3.4.2 Postsqualene sterol biosynthesis in plants

The simplified biosynthetic pathway downstream from cycloartenol is shown in Figure 3.5. Campesterol are 24-methyl, whereas sitosterol and stigmasterol are 24-ethyl analogues of cholesterol. As a surprise for many is cholesterol, besides the 24-methyl and 24-ethyl sterols, also widely present in plants (Heftmann, 1983). Cholesterol typically accounts for 1-2% of the total plant sterols and up to 5% in selected plant families (Moreau et al., 2002). Especially species of *Solanaceae* include high levels of cholesterol (Zygadlo, 1993;Whitaker, 1991; Whitaker, 1988). The proportions of cholesterol and the major plant sterols sitosterol and campesterol are determined by the activity of sterol methyltransferases (SMTs) which catalyze the transfer of two carbon atoms from *S*-adenosyl methionine to make the 24-alkylations (reviewed by Schaller, 2003). There are two classes of SMTs: SMT1 and

SMT2, which are considered important regulatory steps in the biosynthesis of sterols in plants (reviewed by Schaller, 2003). It is unknown how cholesterol is synthesized in plants, but it seems that SMT1, that catalyzes the first methylation of cycloartenol to 24-methylene cycloartenol interfere with the accumulation of cholesterol in plants (Figure 3.5). In *Arabidopsis thaliana* plants bearing a SMT1 knockout is cholesterol the major sterol, composing 26% of the total sterols, compared with 6% in wild-type plants (Diener et al., 2000). The accumulation of cholesterol in plants bearing a SMT1 knockout indicates that the production of high amounts of cholesterol results from a by-pass of SMT1, but this remains to be established.

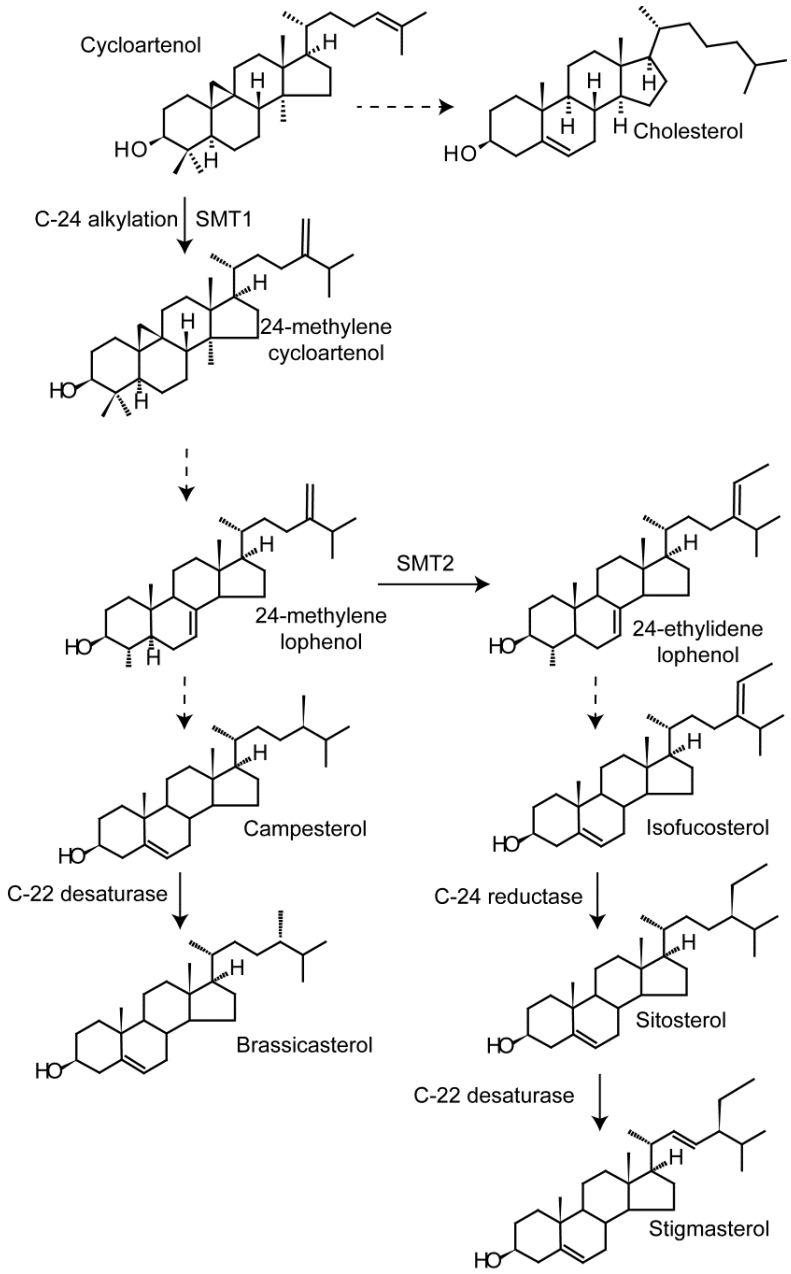

Figure 3.5. Simplified biosynthetic pathway for plant sterols. Dashed arrows indicate more than one biosynthetic step. SMT corresponds to sterol methyltransferase (Paper IV).

4 Summary of papers describing the experimental work

This thesis is based on three research papers covering various aspects regarding vitamin D in plants. A short summary of the papers is given here. The reader is recommended to read the research papers before reading the rest of the thesis.

Paper I. The aim of this study was to develop a new method to determine vitamin D and its sterol precursors. Vitamin D_3, vitamin D_2, 7-dehydrocholesterol, ergosterol, lanosterol, cycloartenol, desmosterol, lathosterol and cholesterol were included in the method. The method was based on saponification and liquid-liquid extraction followed by solid phase clean-up of the compounds from plant leaves and detection by liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS). The method was validated, recoveries ranged from 101% to 114% and precision from 3% to 12%. Detection limits were 2-8 ng/g fresh wt. for the substances tested. A pilot study on *Solanum glaucophyllum* Desf. (*S. glaucophyllum*), *Capsicum annuum* L. (*C. annuum*), *Pisum sativum* L. (*P. sativum*), *Sorghum bicolor* L. (*S. bicolor*) and *Solanum lycopersicum* L. (*S. lycopersicum*) was also described in this paper. Vitamin D_3 was identified in the leaves of *S. glaucophyllum* and *S. lycopersicum* after UVB-exposure. The provitamin D_3, 7-dehydrocholesterol, was identified in the leaves of *C. annuum*, *S. glaucophyllum* and *S. lycopersicum*. No vitamin D_3 was found in either *P. sativum* or *S. bicolor*.

Paper II. The aim of this study was to identify important factors for the formation of vitamin D_2 in grass. The paper describes the content and the seasonal variation of ergosterol and vitamin D_2 in grass. Six varieties of perennial ryegrass (*Lolium perenne* L.) were harvested four times during the season and the content of vitamin D_2 and ergosterol was analyzed by a modified version of the LC-MS/MS method developed in **Paper I**. The average content of vitamin D_2 found was 2 µg/kg, whereas the vitamin D_2 content was maximum 2‰ of the ergosterol content. The content of ergosterol and vitamin D_2 changed more than a factor of 10 during the season. Weather factors were recorded and a principal component analysis was performed to study which factors that were important for the formation of vitamin D_2. This suggested that a combination of weather factors was involved.

Paper III. The aim of this study was to obtain a better understanding of vitamin D_3 synthesis and metabolism in plants and how it changes after UVB- and heat-treatment. A sensitive and selective LC-MS/MS method involving Diels-Alder derivatization was used to identify vitamin D_3 and its hydroxylated metabolites in the leaves of *S. glaucophyllum*, *S. lycopersicum* and *C. annuum*. Vitamin D_3 and $25OHD_3$ were found in all UVB-treated plants. Vitamin D_3 was found in both UVB- and non-UVB-treated plants, but the content of the UVB-treated plants was 18-64 times higher. The highest vitamin D_3 content (200 ng/g dry wt.) was found in UVB-treated *S. glaucophyllum*, which was the only plant that also contained $1,25(OH)_2D_3$ (32 ng/g dry wt.). Enzymatic hydrolysis was used to study the occurrence of glycoside conjugates. These were only found for UVB-treated *S. glaucophyllum* (17 ng/g dry wt.). Cholesterol and 7-dehydrocholesterol were found in all plants and may serve as precursors for vitamin D_3 in plants.

5 Analytical methods

*This chapter describes the relevant background for the choice of analytical methods used in **Paper I, II and III**. Section 5.1 describes traditional methods used for identification of vitamin D. Section 5.2 summarizes the considerations made for choice of detection method. Section 5.3 focuses on development of the LC-APCI-MS/MS method described in **Paper I**. Analysis of the hydroxylated metabolites of vitamin D_3 and the method used in **Paper III** are discussed in Section 5.4. Analysis of glycosides is finally discussed in Section 5.5.*

5.1 Traditional methods for vitamin D

Vitamin D has traditionally been measured by *in vivo* and *in vitro* bioassays (Review by Boland et al., 2003). The accepted official method for vitamin D analysis was for many years the line test using animals (Parrish and Richter, 1979). Either a rat or a chicken was put on a vitamin D deficient diet until the animal developed rickets. After the animal developed rickets, it was fed plant material or extracts and it was estimated how much the plant was able to cure rickets by staining with silvernitrate to show deposition of calcium salts or by radiographic pictures. The line test is time-consuming as it takes approximately five weeks. Results from the biological assays are given in international units (IU), the conversion ratio is 1 IU to 0.025 µg vitamin D. Increased calcium absorption and elevation in blood calcium has also been used as an indicator for the existence of vitamin D-related compounds in plants (Mautalen, 1972; Basudde and Humphreys, 1976;Walling and Kimberg, 1975). The accuracy of these methods may be discussed. The fact is that the amount of quantified vitamin D corresponds to the total vitamin D activity independent of the specific metabolites and their possible difference in activity. Alternative methods were, therefore, used that specifically studied the occurrence of $1,25(OH)_2D$. A high strontium intake by chickens block the conversion of 25OHD to $1,25(OH)_2D$ by suppressing 1α-hydroxylase activity (Wasserman, 1974). The inhibitory effect of strontium can be overcome by the administration of $1,25(OH)_2D$, but not by 25OHD and vitamin D. Studies of calcium absorption in nephrectomized rats, with a suppressed 1α-hydroxylase activity, has also been used (Walling and Kimberg, 1975). However, the accuracy of these methods can be discussed. The biological activity could be due to other compounds that interfere with vitamin D metabolism, calcium absorption or to compounds present e.g., calcium and phosphorus that increase or

inhibit the activity of vitamin D. Consequently, selective chemical methods are needed to study vitamin D and its metabolites in more details.

5.2 Choice of detection method for vitamin D and its sterol precursors

Analysis of vitamin D in plants is not an easy task. Plant material is a complex matrix and the small amounts of vitamin D make quantification even more challenging. Gas chromatography (GC) was the first chromatography principle used to replace the biological assays. Vitamin D needs to be derivatized to trimethylsilyl (TMS) ethers, for analysis by GC, due to poor volatility (Bell and Christie, 1973). Vitamin D undergoes thermal cyclization at temperatures (>125°C) resulting in formation of the corresponding pyro and isopyro compounds (Yeung and Vouros, 1995). Vitamin D gives rise to double peaks as a result of this thermic rearrangement (Yeung and Vouros, 1995). Because of a decrease in single ion monitoring (SIM) sensitivity by cyclization and the need of a derivatization step did GC methods not gain use for routine assays. However, some early studies on vitamin D in plants did use GC for identification (Rambeck et al., 1979;Suardi et al., 1994).

Currently, most analytical methods for vitamin D in food are based on high performance liquid chromatography (HPLC) with UV detection (265 nm). The purity of the peak is typically evaluated with a photo diode array detector (DAD) 220-320 nm. HPLC methods rely on multiple chromatographic purification steps and are generally both sensitive and repeatable (Byrdwell, 2009). However, these methods are quite laborious due to the need of many clean-up steps to achieve a satisfactory selectivity by UV/DAD detection. Analysis of vitamin D in complex matrices like plants can be especially problematic because of a high degree of co-eluting interferences. Figure 5.1 shows an UV chromatogram of an *Arabidopsis thaliana* extract spiked with vitamin D_3. The chromatogram is complex and a lot of interfering peaks present even after extensive sample clean-up. Two semi-preparative HPLC steps were included besides solid phase extraction (SPE). One with a silica and an amino column connected in series (Jakobsen et al., 2004) and one with a cyano column (Jakobsen et al., 2007). This demonstrates the shortcomings of UV detection. Despite its drawbacks has HPLC with UV detection been used for analysis of vitamin D in plants (Aburjai et al., 1998;Curino et al., 1998;Prema and Raghuramulu, 1996; Prema and Raghuramulu, 1994;Aburjai et al., 1996;Curino et al., 2001;Aburjai et al., 1997).

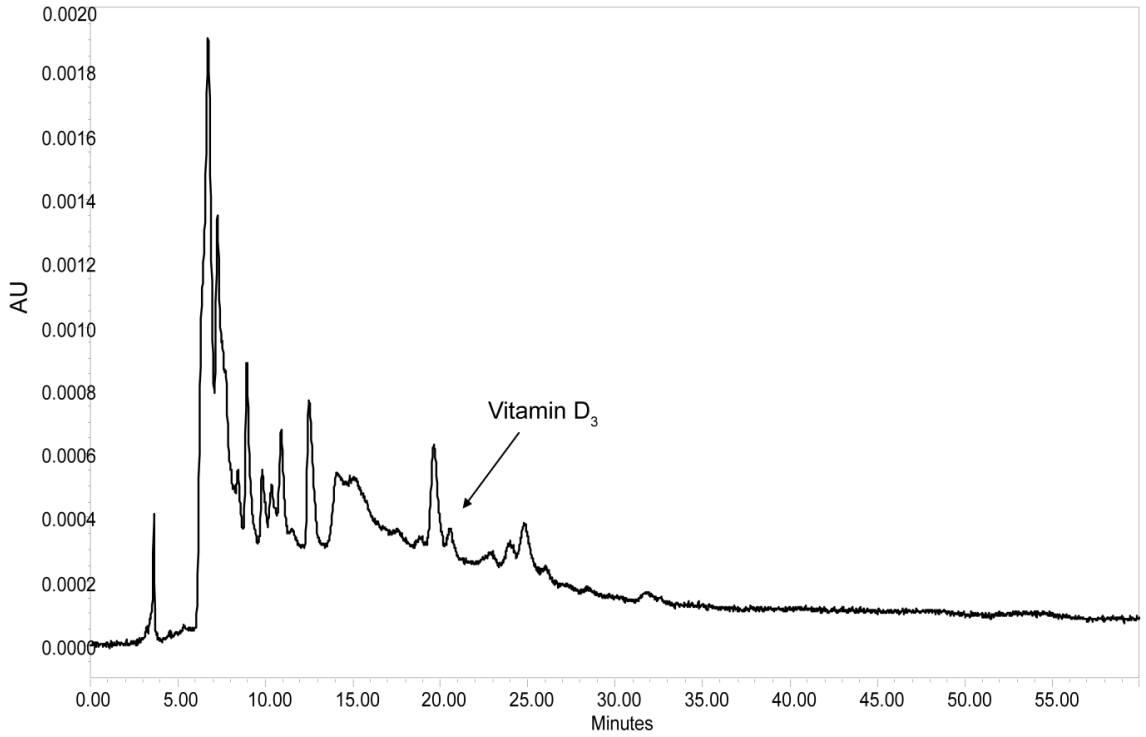

Figure 5.1. UV chromatogram (265 nm) for an *Arabidopsis thaliana* extract spiked with vitamin D_3. The peak of interest is labeled with an arrow.

In the present study, UV detection was replaced by mass spectrometry (MS) detection in order to improve selectivity (**Papers I, II and III**). Limitations caused by incomplete resolution of different molecular species in a complex mixture can be resolved by coupling HPLC with MS. MS has been used in a number of previous studies for identification of vitamin D in plants, but not coupled to HPLC (Curino et al., 1998;Skliar et al., 2000;Aburjai et al., 1996). Nuclear magnetic resonance (NMR) would offer valuable information in addition to UV and MS detection. NMR is a powerful tool for structure elucidation and can discriminate between compounds that only differ in terms of local chemical environment, e.g. compounds with identical masses, but different locations of functional groups. However, in general NMR analyses lack sensitivity of the mass spectrometer and more purified samples are often needed (Eisenreich and Bacher, 2007). Nevertheless, ^{1}H NMR has been used for identification of vitamin D_3 in plants, even though extraction of two kg fresh plant leaves was required (Aburjai et al., 1998).

Sterols act as precursors of vitamin D and sterol analysis is, therefore, essential to investigate the biosynthesis of vitamin D in plants. Sterols are typically measured as TMS ether derivates by GC (Piironen et al., 2000), the detection principle used can be either flame ionization

(FID) (Brufau et al., 2006;Liu et al., 2007;Phillips et al., 2005) or MS (Nyström et al., 2007; Toivo et al., 2001). MS is in general an important technique for identification of sterols and for evaluating peak purities. GC has the disadvantage that derivatization is required, which may lead to reduced recovery and the formation of artifacts (Razzazi-Fazeli et al., 2000;Lu et al., 2007). HPLC offers compared to GC the advantage of analysis without derivatization and more gentle conditions suitable for thermally unstable sterols. Detection can be carried out by UV (200-210 nm) (Careri et al., 2001;Sanchez-Machado et al., 2004), evaporative light scattering (ELS) (Hong et al., 2007) and MS (Lu et al., 2007;Mezine et al., 2003;Ruibal-Mendieta et al., 2004;Rozenberg et al., 2003;Cañabate-Díaz et al., 2007). Sterols only adsorb UV in the 200-210 nm range and low sensitivity is, therefore, observed by UV detection due to low transparency of most organic solvents. Furthermore, UV detection is non-specific for sterols and a complete separation is needed. GC is generally considered superior over HPLC for sterol analysis (Lagarda et al., 2006), but progress in HPLC technology has occurred recent years. Columns with particle sizes of 1-2 μm have been introduced which may improve resolution of co-eluting sterols (Lu et al., 2007).

It was desirable to choose a detection method that could determine vitamin D and its sterol precursors in the same run and liquid chromatography tandem mass spectrometry (LC-MS) was, therefore, selected. The original goal was to develop a rapid method suitable for high throughput analysis of a fairly large amount of samples in connection with a breeding program. Consequently, the initially aim was to develop a screening method to study sterols, vitamin D and its hydroxylated metabolites with minimum sample preparation using one analytical method. This could be achieved with application of techniques known from metabolomics and metabolic profiling studies. Time of flight mass spectrometry (TOF-MS) is well suited for this purpose because of a high mass resolution (>10,000 at full width half maxima) and high mass accuracy (<3 ppm) (Williamson and Bartlett, 2007). TOF-MS was, therefore, exploited for identification of vitamin D and related compounds in plants. This was complicated by ion suppression and adduct-formation upon ionization of the crude extracts, which made limit of detection high (1 μg/mL). The content of vitamin D in plants is in pg-ng range and it was, therefore, concluded that a screening method not could provide the sensitivity needed. Tandem mass spectrometry (MS/MS) using a triple quadrupole instrument was chosen as the detection principle on the basis of these preliminary studies.

5.3 Analysis of vitamin D_3 and its sterols precursors

*A LC-MS/MS method using atmospheric pressure chemical ionization (APCI) was developed for analysis of vitamin D and its sterol precursors (**Paper I**). The sample preparation procedure is discussed in section 5.3.1, followed by a discussion of suitable ionization methods in Section 5.3.2 and LC-MS/MS principles in Section 5.3.3. The complete description of the method is given in **Paper I**.*

5.3.1 Sample preparation

Triple quadrupole instruments typically have unit mass resolution and low mass accuracy (100 ppm). As a result sample extracts has to be relatively free from interferences, which makes proper sample preparation crucial. Furthermore, inherent enzymatic activity in the plants has to be rapidly stopped. Inactivation was achieved by freeze-drying of all samples, as enzymes and transporters are unable to work in absence of water (Fiehn, 2002). Following freeze-drying were the samples homogenized into a fine powder in a conventional blender. Precaution has to be taken to prevent decomposition of vitamin D by exposure to light and oxidation. The laboratory must have an UV absorbing film on the windows or similar precautions must be taken (CEN, 2008). Sodium ascorbate was used as an antioxidant during extractions along with nitrogen-flushing to prevent oxidation.

The analysis of sterols and vitamin D typically begins with saponification (alkaline hydrolysis) to liberate ester-bound forms. Triglycerides are hydrolyzed into glycerol and fatty acids thereby leaving the free forms in the non-saponified matter. Saponification can be either hot or cold, but cold is preferred due to reversible and temperature-dependent equilibration between vitamin D and previtamin D (Buisman et al., 1968;Hanewald et al., 1968;Schlatmann et al., 1964). The extraction of free sterols and vitamin D present in the non-saponified matter is usually performed by liquid/liquid extraction using rather non-polar organic solvents (CEN, 2008). In the present study, pentane:ethylacetate (80:20) and *n*-heptane:ethylacetate (80:20) were used (**Papers I, II and III**). Purification of the crude extracts is typically needed, this is necessary both to avoid contamination of the analytical column and the ionization source by interfering substances e.g., chlorophyll and other lipophilic pigments (**Paper I**). Purification can be achieved by thin layer chromatography (TLC), column chromatography, semi-preparative HPLC or more recently by solid phase extraction (SPE). Fractionation with TLC

and column chromatography is time-consuming and not suitable for routine analysis and can usually be replaced by SPE providing faster fractionation using less solvent (Lagarda et al., 2006). Consequently, a silica SPE clean-up step was included in the method (**Paper I**).

The use of internal standard is essential for quantification of vitamin D due to the aforementioned reversible isomerization with the corresponding previtamin D. An internal standard is in addition used to eliminate analytical errors due to: 1) loss of sterols/vitamin D during extraction and separation and 2) signal variation during ionization. Vitamin D_2 and vitamin D_3 are chemically very similar and vitamin D_2 has been used as the internal standard when determining vitamin D_3 and *vice versa*. However, this is not the best approach when vitamin D_2 and vitamin D_3 occur simultaneously as could be the case in plants (Horst et al., 1984). Deuterium-labeled compounds are ideal internal standards for quantification by MS because of the complete resemblance with the analyte. Consequently, deuterated standards were used for quantification of vitamin D and its metabolites (**Papers I, II and III**). Deuterated sterols are either very expensive or non-available. Deuterated vitamin D was, therefore, used as internal standard for the sterols, whereas deuterated cholesterol functioned as an instrument standard to account for signal variation during ionization.

5.3.2 Ionization techniques

Conversion of analytes to gas phase ions is essential for any MS analysis to work and a main limitation in MS is as consequence low ionization efficiency. The most used ionization source for LC-MS is electrospray ionization (ESI). ESI is a soft ionization technique, which means that it transfers ions from solution to gas phase without breaking any chemical bonds. ESI can be divided into three stages: 1) formation of charged droplets by an electrical capillary 2) solvent evaporation and droplet fission and 3) formation of gas-phase ions. ESI works best when the analyte already is in its ionic form in solution (Cech and Enke, 2001) and the ionization efficiencies of vitamin D and its sterol precursors are consequently low in ESI because of few polar functional groups (Dimartino, 2007). Atmospheric pressure chemical ionization (APCI) is another soft ionization technique, which is more used for vitamin D analysis. The great advantage of APCI is that it, different from ESI, actively generates ions from neutrals. The droplets from the LC inlet are in comparison to ESI not charged. The APCI source contains a heated vaporizer, which facilitates rapid vaporization of the droplets.

Gas-phase electrons are provided by a corona discharge needle and solvent molecules are ionized first, followed by gas-phase ion-molecule reactions with analytes (Gao et al., 2005). Consequently, APCI is a much better ionization technique for neutral and apolar substances such as vitamin D and sterols. A comparison between ESI and APCI mode was made (Figure 5.2A and Figure 5.2B, respectively). The sensitivity of vitamin D_3 was 25 times higher in APCI than ESI, when a 1 µg/mL standard mixture was used. Sterol sensitivity was particularly poor (Figure 5.2A). Useful results were only acquired in positive mode for both ESI and APCI.

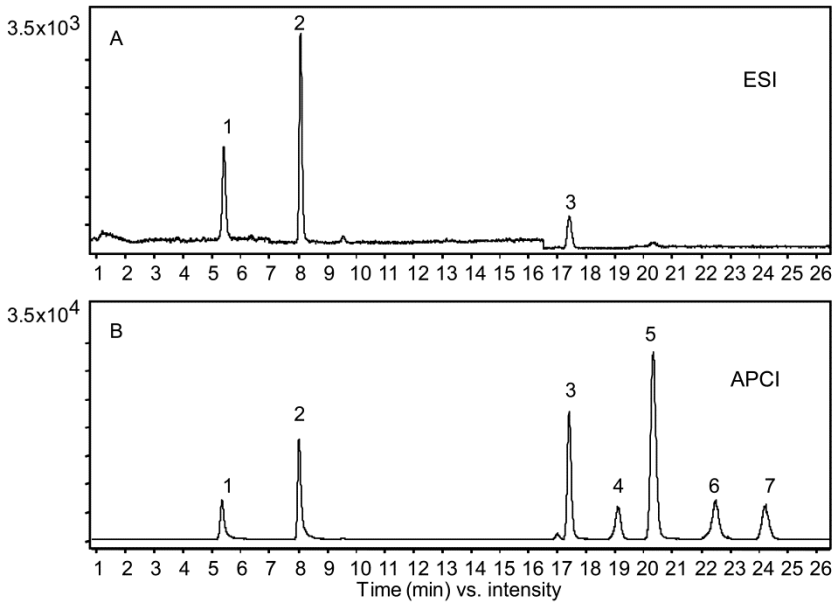

Figure 5.2. (A) ESI-MS chromatogram and (B) APCI-MS chromatogram of a 1 µg/ml standard mixture of 1) $1,25(OH)_2D_3$, 2) $25OHD_3$, 3) Vitamin D_3, 4) Desmosterol, 5)7-dehydrocholesterol, 6) Cholesterol and 7) Cycloartenol.

Atmospheric pressure photoionization (APPI) is another soft ionization technique suitable for lipophilic compounds. The ionization process in the APPI ion source is started by UV light, similar to corona discharge in APCI. The principle used is based on UV irradiation of the vaporized effluent of the HPLC column inside the ion source, which initiates a cascade of gas-phase reactions leading to ionization of the analyte (Kushnir et al., 2010). APPI typically requires a dopant, which is an additive that must be photoionizable. The most commonly used dopants are toluene and acetone. First, the dopant molecules are ionized directly by the UV photons, which is advantageous because dopant molecules far outnumber the analyte molecules and more collisions then results in formation of an ion. The dopant ion can then donate a proton to the analyte molecule. The use of dopant usually requires an additional pump, which complicates method development. Consequently, positive mode APCI was concluded to be the most suitable ionization technique (**Paper I**).

5.3.3 LC-MS/MS

MS/MS is typically made on a triple quadrupole instrument. The quadrupole instrument consists of two quadrupole mass filters and a collision cell (Figure 5.3). The sensitivity of the triple quadrupole is low in full scan, better in selected ion monitoring (SIM) mode and highest in selected reaction monitoring (SRM) mode (Figure 5.3). In SRM the precursor ion is selected in the first quadrupole mass filter and passed into the collision cell, which is filled with nitrogen. The selected precursor ion is fragmented and a few product ions are selected for detection in the second mass filter (Figure 5.3). In this way is background noise not visible in the chromatograms resulting in a good signal to noise ratio. SRM is, therefore, justified by the need to achieve low detection limits without interferences. SRM increase selectivity, but more than one transition is needed for reliable confirmation of a specific analyte, which preferable is combined with other evidence such as relative intensities of product ions in the mass spectra, retention time and peak shape positively to identify the compound as vitamin D (**Paper I**).

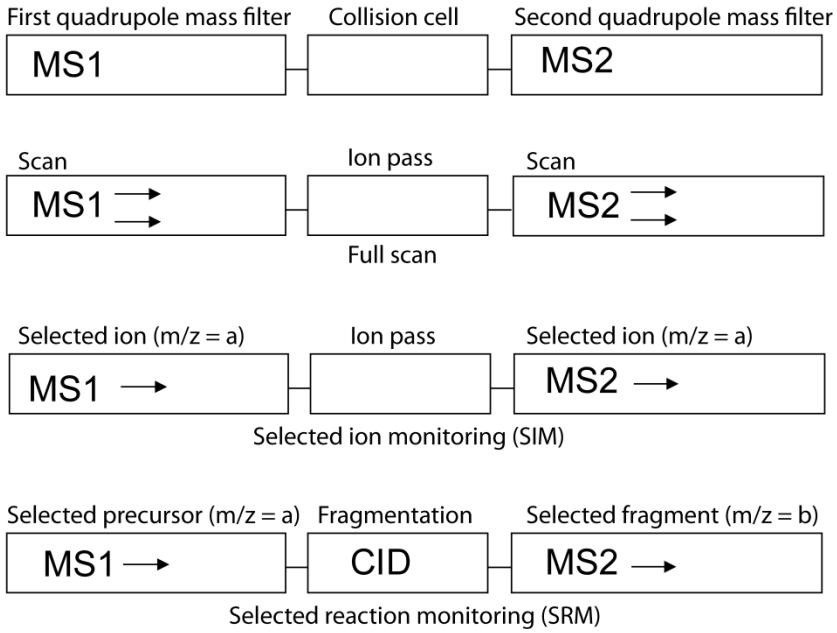

Figure 5.3. Scan modes in a triple quadrupole instrument, CID refers to collision induced dissociation.

MS offers an additional level of resolution by differentiating co-eluting compounds by mass. However, many sterols equal in mass and the small differences in the sterols side chain lead to challenges in obtaining an acceptable separation. The aim was to develop a gradient method that could resolve related isobaric sterols in the plant extract. Various reversed phase columns were tested and a PFP (pentafluorophenyl) column was chosen over conventional

C18 and C8 columns. The PFP material is less hydrophobic than the C18 and C8 material and faster separation using less organic solvent is, therefore, obtained. Baseline resolution of all compounds was not achieved, but the selectivity of the SRM acquisition made it possible to separate all the analytes within 13 minutes giving a total run time of 28 minutes including cleaning and reequilibration of the column (**Paper I**).

5.4 Analysis of hydroxylated metabolites of vitamin D_3

*The complete analytical procedure used for analysis of the hydroxylated metabolites is described in **Paper III**. The choice of detection method is discussed in Section 5.4.1, whereas considerations regarding method development are given in Section 5.4.2.*

5.4.1 Choice of detection method

The hydroxylated metabolites of vitamin D_3 represent a challenge because they exist in even lower concentrations than vitamin D_3. They have been detected in plants using both protein-binding assays (Skliar et al., 2000;Curino et al., 2001) and traditional analytical methods (Aburjai et al., 1998;Prema and Raghuramulu, 1996;Prema and Raghuramulu, 1994;Aburjai et al., 1996). The most common chemical detection principle used has been HPLC with UV detection, which as mentioned in section 5.2, is not totally specific. Protein-binding assays, including RIA (radioimmunoassay) and RRA (radioreceptor binding assay), is widely used for analysis of 25OHD and $1,25(OH)_2D$ in clinical laboratories due to the simplicity (Hollis and Horst, 2007). Protein-binding assays consist of the displacement of a bound labeled substrate from its receptor by an unlabeled substrate. Increasing amounts of the unlabeled substrate lead to an increase in free labeled substrate that can be measured and compared to a standard curve. RIA's for both $1,25(OH)_2D$ and 25OHD exist, these assays are commercialized and are widely used especially for serum samples (Hollis and Horst, 2007). RIA has been used without any prior purification on extracts and cell cultures of *S. glaucophyllum* and *C. diurnum* (Weissenberg et al., 1988). However, the lipophilic nature of vitamin D makes it difficult to analyze in any protein-binding assay due to solubility problems (Hollis and Horst, 2007). Matrix effects are also common, usually due to lipids not found in the standard tube, but in the assay tube that competes with binding to the protein (Hollis and Horst, 2007). Moreover, equal detection of $25OHD_2$ and $25OHD_3$ represents a challenge, in particular for assays based on DBP, because binding proteins from many species

show higher affinity for $25OHD_3$ than for $25OHD_2$ (Singh et al., 2006). It is discussed whether the biological activity of the two forms is the same (section 2.3.1) and it is, therefore, important to be able to discriminate. However, RIA could differentiate among lower and higher yielding plants in a screening process. This was demonstrated by Gil et al. (2007) who used RIA for screening of vitamin D activity in *S. glaucophyllum*. RRA was developed in the middle 1970s, where the VDR was harvested from chicken duodena mucosa (Eisman et al., 1976;Brumbaugh et al., 1974) and later calf thymus (Hollis, 1986). RRA is performed with ^3H-$1,25(OH)_2D_3$ that competes for binding to VDR. RRA is highly sensitive, but besides the obvious disadvantage of isolating VDR from animals, the possibility of non-specific competition or inactivation of VDR by other lipophilic compounds has to be taken into account. RRA has been applied for identification of $1,25(OH)_2D_3$ in *S. glaucophyllum* (Curino et al., 2001) and *Nicotiana glauca* (Skliar et al., 2000).

Protein-binding assays are currently being replaced by MS methods due to their higher specificity and especially detection of 25OHD in serum by LC-MS/MS is widely used (El-Khoury et al., 2010). However, direct LC-MS/MS analysis is challenging because of poor ionization efficiency, low concentration and an extensive product ion spectra. Attempts to increase ionization efficiency of vitamin D_3 metabolites have been reported several times mostly for serum samples. Kissmeyer and Sonne (2001) developed a LC-MS/MS method that quantified the ammonium adduct of $1,25(OH)_2D$ with a LOD of 20 pg/mL using 1 mL of serum. Another LC-MS/MS method quantifying the lithium adduct of $1,25(OH)_2D$ with an LOD of 15 pg/mL using a 0.2 mL serum sample has also been developed (Casetta et al., 2010), but included a complicated LC system. Derivatization with Cookson-type reagents has often been employed to enhance the detection response in MS (Aronov et al., 2008;Gao et al., 2005;Higashi and Shimada, 2004;Higashi et al., 2011;Kamao et al., 2007). Aronov et al. (2008) used 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), a Cookson-type reagent, to derivatize $1,25(OH)_2D$ before LC-ESI-MS/MS analysis and a LOD of 25 pg/mL using 0.5 mL of serum was achieved. Microflow LC-MS together with derivatization has also been used, which improved sensitivity 15-fold compared to normal LC when using a 0.2 mL serum sample (Duan et al., 2010). However, microflow LC has a small loading capacity that counteracts the sensitivity gain, especially when analyzing complex matrices (Duan et al., 2010). Derivatization with PTAD followed by detection with LC-ESI-MS/MS was chosen for

analysis of hydroxylated metabolites of vitamin D in plants because high sensitivity can be achieved using a conventional LC-MS/MS instrument.

5.4.2 Development of method to study hydroxylated metabolites

A LC-ESI-MS/MS method using Diels-Alder derivatization with PTAD was developed to study the hydroxylated metabolites of vitamin D_3 (**Paper III**). PTAD reacts with the *s*-cis-diene in vitamin D_3 by a Diels-Alder reaction, which by introducing a proton-accepting amide group favor positive-mode ionization and in the same time shifts the molecular weight to a higher mass range, where background noise is lower. Additionally, the product ion spectrum of derivatized vitamin D_3 metabolites exhibit only one major fragment i.e., *m/z* 298 for $25OHD_3$ (Figure 5.4B), which is beneficial for sensitive SRM analysis. In comparison, non-derivatized vitamin D_3 metabolites produce very rich product ion spectrums due to many low-energy fragmentation pathways, which hampers sensitivity (Figure 5.4A). Derivatization typically results in a 100-1000 fold increase in sensitivity over non-derivatized compounds (Aronov et al., 2008). A further increase in ionization efficiency was achieved in **Paper III** by addition of methylamine to the mobile phase giving rise to the $[M+CH_3NH_3]^+$ adduct ion (Ding et al., 2010;Higashi et al., 2008).

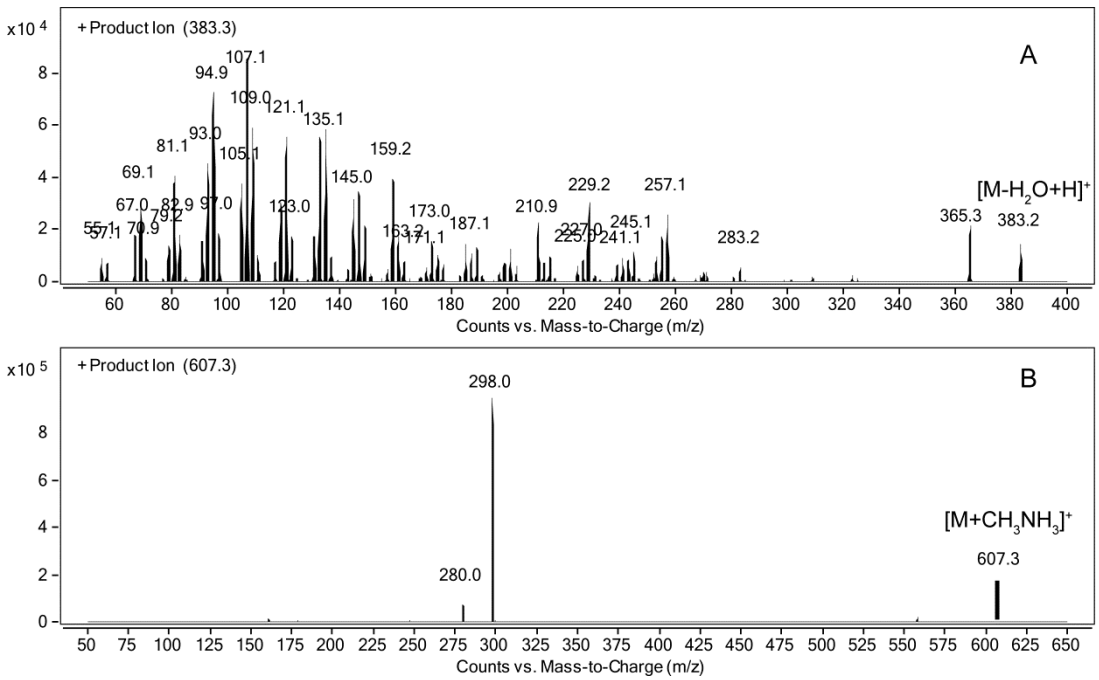

Figure 5.4. ESI product ion spectrum of (A) Non-derivatized $25OHD_3$ and (B) Derivatized $25OHD_3$.

Many Diels–Alder reactions are reversible and the decomposition reaction of the cyclic system is then called a retro-Diels-Alder reaction. This is commonly observed when a Diels-Alder product is analyzed by MS. However, fragmentation of the vitamin D derivate does not involve a retro-Diels-Alder reaction (Weiskopf et al., 2001). The derivatization product and the fragmentation product ion for $25OHD_3$ can be seen in Figure 5.5.

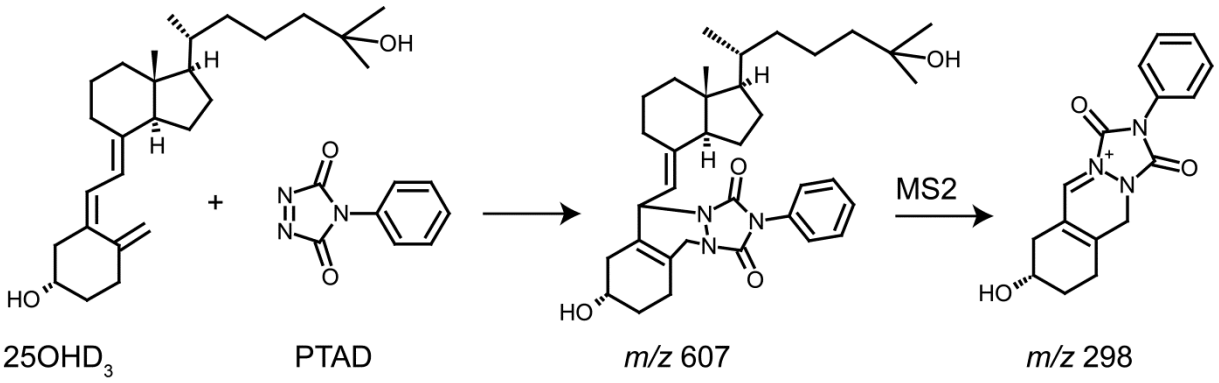

Figure 5.5. Derivatization of $25OHD_3$ with PTAD and fragmentation product ion.

Two epimers, 6*S* and 6*R*, are produced by derivatization because PTAD reacts with the *s*-cis-diene system from both the α- and β-side (Ding et al., 2010;Higashi et al., 2001). The ratio between 6*S* and 6*R* is about 4:1 and two peaks are, therefore, expected for each metabolite (Ding et al., 2010). Only the $25OHD_3$ epimers were fully separated and the major peak for the 6*S*-isomer was used for quantification (**Paper III**). Vitamin D_3 and $1,25(OH)_2D_3$ eluted as one peak, which increased sensitivity, but also broaden the peaks (**Paper III**).

The hydroxylated metabolites have higher polarity than vitamin D_3. However, despite the difference in polarity can vitamin D_3 and $25OHD_3$ be extracted in the same run (Mattila et al., 1995a;Jakobsen et al., 2004). The extraction of $1,25(OH)_2D_3$ turned out to be problematic and low recovery (10%) was experienced due to poor extraction efficiency from the non-saponifiable matter. Despite this, the procedure was chosen to study all vitamin D related metabolites in the limited plant material which could be produced. Only few studies have included quantification of $1,25(OH)_2D_3$ in food in general (Montgomery et al., 2000; Takeuchi et al., 1988;Kunz et al., 1984). The extraction process used differed from the standard procedure for vitamin D_3 and $25OHD_3$ as no saponification was included. The possibility that this will extract total $1,25(OH)_2D_3$ is questionable since no data are available for the level of conjugated forms of $1,25(OH)_2D_3$. It needs to be investigated whether $1,25(OH)_2D_3$ is acetylated or bound to other food components.

5.5 Analysis of glycosides

Saponification liberates esters and acetylated glycosides as glycosides, but fails to hydrolyze the bond between vitamin D and the carbohydrate moiety (Toivo et al., 2001). The initial aim was, therefore, to develop an analytical method to study glycosylated forms. The final procedure applied to plants is described in ***Paper III****, while this section describes the reason for this choice.*

5.5.1 Direct analysis

A mixed steryl glycoside standard from Matreya LLC (Pleasant Gap, PA, USA) was used as a surrogate during method development as no commercial available vitamin D_3 conjugates exist. Several methods have been described for analysis of steryl glycosides in various plant matrices and both direct and indirect analysis (with or without hydrolysis) has been used (Van Hoed et al., 2008). Direct analysis is advantageous because a sample preparation step is avoided and was tested first. Direct steryl glycoside analysis can be performed with GC-FID (Phillips et al., 2005;Lacoste et al., 2009), HPLC-UV (Kesselmeier et al., 1985), HPLC-ELSD (Moreau et al., 2008), GC-MS (Gutiérrez and José, 2001) and LC-MS (Rozenberg et al., 2003). The present study used LC-MS/MS due to a superior sensitivity and selectivity in comparison with other detection techniques. The experiments were carried out using a Quattro Premier Tandem mass spectrometer (Waters, Milford, MA). The behavior of the steryl glycosides were examined using both negative and positive ESI, APCI and APPI. The objective was to find the most suitable ionization technique to produce intense structure specific product ions. Positive APPI-MS turned out to be the most promising technique. The parameters used can be seen in Table 5.1.

Table 5.1. Parameters used in positive APPI-MS/MS
Argon was used as a collision gas

Parameter	
Cone gas flow	600 L/h
Desolvation gas flow	115 L/h
Source temperature	120°C
Probe temperature	650°C
Collision gas pressure	4.0×10^{-3} mbar
Collision energy	45 V
Cone voltage	55 V

The solvent used was a mixture of isopropanol:water:methanol (10:15:75). Isopropanol was used to promote photoionization instead of a dopant, which is called solvent photoionization. The steryl glycosides formed very strong and stable adducts with sodium. A product ion scan of the sitosteryl glycoside sodium adduct can be seen in Figure 5.6.

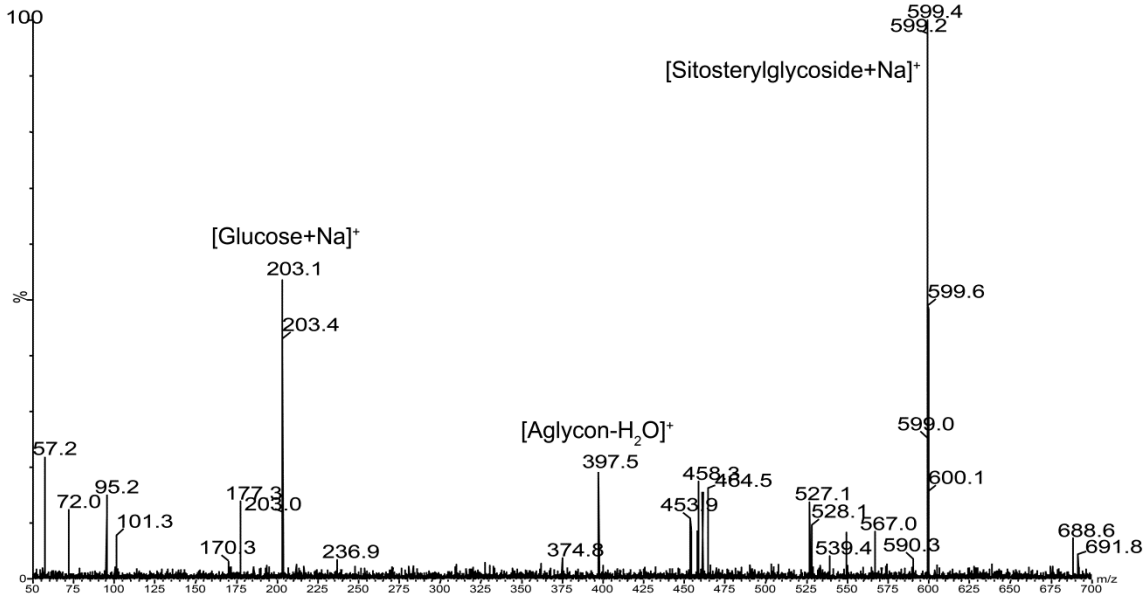

Figure 5.6. Product ion scan of the major ion for sitosterylglycoside (*m/z* 599.4) obtained by APPI-MS.

The main product ion was $[Glucose+Na]^+$, whereas high collision energies was needed to produce the $[Aglycon-H_2O]^+$. Consequently, direct analysis of sitosteryl glycoside is possible, as $[steryl\ glycoside+Na]^+ \rightarrow [Glucose+Na]^+$ (*m/z* 599.4 > *m/z* 203.1) could be chosen as SRM transition or at high collisions energies $[steryl\ glycoside+Na]^+ \rightarrow [Aglycon-H_2O]^+$ (*m/z* 599.4 > *m/z* 397.5). Similar transitions could probably be seen for vitamin D glycosides. However, no vitamin D glycoside standards are commercially available and the diversity of the existing forms makes direct analysis complicated. Thus, it was concluded that hydrolysis prior to analytical determination was a better approach.

5.5.2 Hydrolysis

Acid hydrolysis has traditionally been used to release glycosidic sterols (Liu et al., 2007; Nyström et al., 2007;Toivo et al., 2001). Acid hydrolysis is typical performed at relatively harsh conditions e.g., 60 minutes at 80°C with 6 M ethanolic hydrochloric acid solution (Nyström et al., 2007;Toivo et al., 2001;Kamal-Eldin et al., 1998) and has the disadvantage that isomerization occurs (Kamal-Eldin et al., 1998). Consequently, the stability of vitamin D_3

and 7-dehydrocholesterol towards acid hydrolysis was investigated. Equal amounts (250 ng) of vitamin D_3 and 7-dehydrocholesterol were added to ten glass tubes (single determination). Acid hydrolysis was performed in these tubes by adding 6 M ethanolic hydrochloric acid solution followed by vortexing. The tubes were finally placed in a hot cupboard at 80°C. A tube was cooled down on ice and neutralized with 6 M aqueous sodium hydroxide at the following time points: 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes. The remaining vitamin D_3 and 7-dehydrocholesterol was extracted and analyzed with the method described in **Paper I**. Extensive degradation of both vitamin D_3 and 7-dehydrocholesterol was observed (Figure 5.7). Thus, acid hydrolysis cannot be applied to quantify either glycosides of vitamin D_3 or 7-dehydrocholesterol.

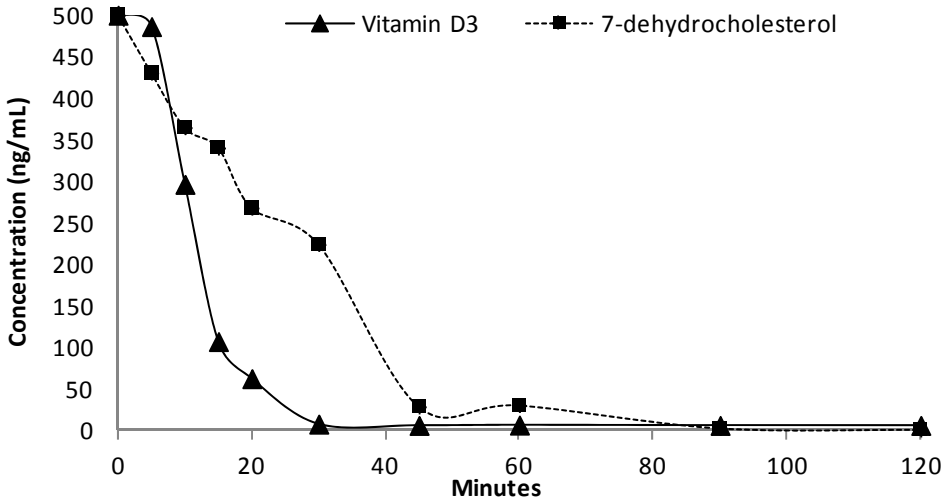

Figure 5.7. Degradation of vitamin D_3 and 7-dehydrocholesterol during incubation with 6 M ethanolic hydrochloric acid at 80°C.

An usually gentler method is enzymatic hydrolysis and this was, therefore, tested. The mixed steryl glycoside standard from Matreya LLC was again used as surrogate. Only few studies have applied enzymatic hydrolysis for steryl glycosides and these were used as a starting point (Kesselmeier et al., 1985;Nyström et al., 2008;Moreau and Hicks, 2004). A cellobiase from *Aspergillus Niger* and a purified β-glucosidase from almonds were tested. The cellobiase was most effective as also observed by Nyström et al. (2008). A combination of the two enzymes was also tested and did not result in a larger degree of hydrolysis than cellobiase alone. Cellobiase in a concentration of 45 units/mL in 0.1 M citric acid buffer solution (pH 5.0) was observed to be most effective. Incubation conditions were optimized to get the largest degree of hydrolysis and corresponding liberation of free sterols. One disadvantage

when using enzymatic hydrolysis for lipohilic compounds is poor solubility in an aqueous solution. Consequently, DMSO and sodium taurocholate were used as solubilizing agents.

The enzymatic hydrolysis procedure used to study the occurrence of vitamin D glycosides is described in **Paper III**. However, this procedure did not result in complete hydrolysis as also described by Nyström et al. (2008). Kesselmeier et al. (1985) has used β-glucosidase in the hydrolysis of steryl glycosides, but recent studies have not been successful in similar hydrolysis (Nyström et al., 2008;Moreau and Hicks, 2004). The enzymatic hydrolysis observed by Kesselmeier et al. (1985) may be due to impurities of minor hydrolases rather than the actual β-glucosidase, whereas similar secondary activities not are present in modern highly purified enzyme preparations (Nyström et al., 2008;Moreau and Hicks, 2004). The active enzyme needs to be identified to fully replace acid hydrolysis, but for vitamin D glycosides is enzymatic hydrolysis the only choice as long as conjugated standards are lacking.

6 Vitamin D in plants

*The occurrence of vitamin D_2 and ergosterol in grass is discussed in Section 6.1 with references to **Paper II**, whereas the occurrence of vitamin D_3, its sterol precursors and its metabolites will be discussed in Section 6.2 with references to **Paper I** and **Paper III**. Biosynthesis of vitamin D_3 will finally be discussed in Section 6.3.*

6.1 The occurrence of vitamin D_2 and ergosterol in grass

One aim of the present PhD thesis was to study the occurrence and seasonal variation of vitamin D_2 and ergosterol in grass. The major part of this work is described in **Paper II**. However, a preliminary study was conducted to estimate the level of ergosterol and vitamin D_2 in grass. The preliminary study is not included in **Paper II**, but a short summary is presented here.

6.1.1 Preliminary study on occurrence of vitamin D_2 and ergosterol in grass

The grasses included in the preliminary study were harvested on 25th of June 2009. The grasses were 30 variants of *Trifolium pretense* L.*, Trifolium repens* L.*, Medicago sativa* L.*, Lolium perenne* L.*, Lolium x boucheanum* Kunth*, Festulolium, Festuca pratensis* Huds.*, Phleum pretense* L.*, Dactylis glomerata* L.*,* and *Poa pratensis* L. The grasses were freeze-dried, homogenized well in a blender and analyzed with the method described in **Paper I**. A sample size of 0.4 g freeze-dried grass was used for analysis. The content of ergosterol was between 70-1480 µg/kg fresh wt. (Appendix), but as a surprise was no vitamin D_2 identified.

An experiment was conducted to investigate whether there was vitamin D_2 in the samples, but below limit of detection (2 µg/kg fresh wt.). *Trifolium repens* (Klondike) and *Trifolium repens* (Rivendel) were selected for further analysis. A larger sample size (3 g) was used and vitamin D_2 was analysed by the HPLC UV/DAD method described by Jakobsen et al. (2004). This method uses a larger extraction volume and a larger SPE column, which makes it possible to weigh in a larger sample size. Two semi-preparative HPLC steps were included besides SPE. One with a silica and an amino column connected in series (Jakobsen et al., 2004) and one with a cyano column (Jakobsen et al., 2007). However, interferences were still observed in the UV chromatogram, despite the use of two semi-preparative HPLC steps

(Figure 6.1). The identity of vitamin D_2 in the purified extract was confirmed with both DAD (Figure 6.1) and with LC-APCI-MS/MS (not shown).

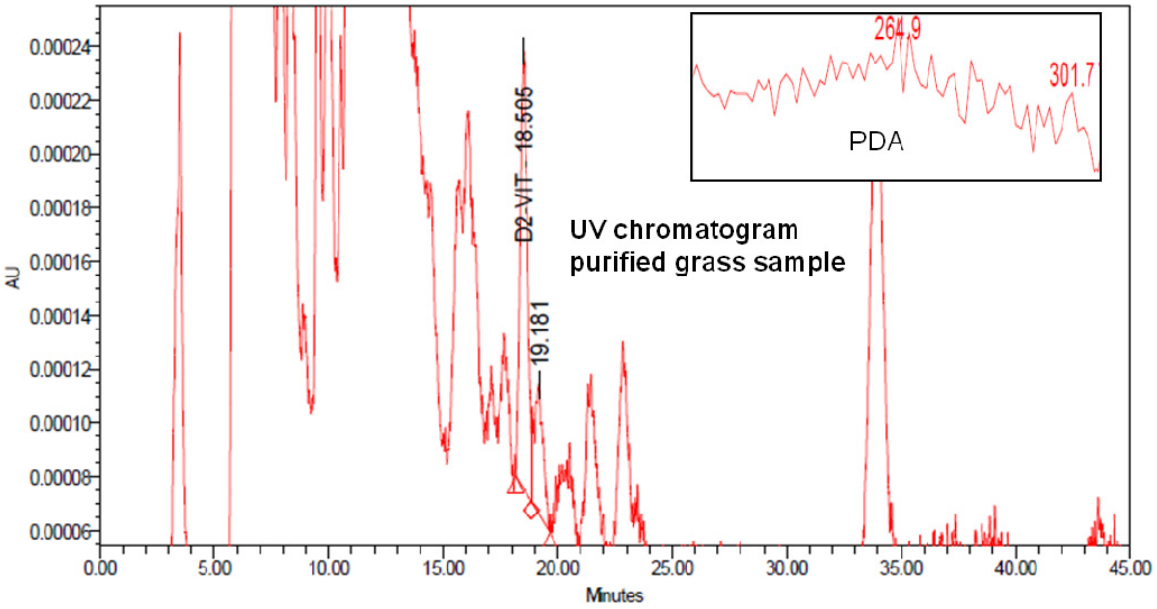

Figure 6.1. UV chromatogram (265 nm) and photo diode array spectrum for vitamin D_2 in the *Trifolium repens* (Klondike) sample.

The content of vitamin D_2 was 0.8 µg/kg fresh wt. for *Trifolium repens* (Klondike) and 0.4 µg/kg fresh wt. for *Trifolium repens* (Rivendel), which is below 2 µg/kg fresh wt. that is the limit of detection of the method described in **Paper I**. It demonstrates that a larger sample size is needed to obtain a reasonable limit of detection. The ergosterol content found in the preliminary study was in comparison 900 µg/kg fresh wt. *Trifolium repens* (Klondike) and 1480 µg/kg fresh wt. for *Trifolium repens* (Rivendel) (Appendix), which is more than thousand times that of vitamin D_2. This makes quantification of both compounds in one run extremely difficult, and it was concluded that a semi-preparative HPLC step was necessary to fractionate the extracts.

Vitamin D activity of grass and hay was studied intensively 50 to 80 years ago using biological methods (Thomas and Moore, 1951;Thomas, 1952;Wallis, 1939;Wallis, 1938;Wallis et al., 1958;Russell, 1929;Newlander and Riddell, 1952;Newlander, 1948;Henry et al., 1958;Steenbock et al., 1925;Moore et al., 1948;Keener, 1954). Most of these studies were on alfalfa (*Medicago sativa* L.) and most of the grasses showed activity. The vitamin D activities ranged from 0-3800 IU/kg, equivalent to 0-95 µg vitamin D/kg. The average vitamin D activity found was about 25 µg/kg, which is higher than obtained in the present

study. This suggests a slight overestimation of vitamin D in previous studies using biological assays, if we presume that no natural decline occurred during the last 50 to 80 years.

6.1.2 Main study in vitamin D_2 and ergosterol in grass

The main grass study was conducted the following year. The aim of this study was to investigate the seasonal variation of vitamin D_2 and ergosterol in grass. A detailed description of this study and the method used is given in **Paper II**. It was concluded, based on the preliminary study, that small modifications of the extraction procedure described in **Paper I** was needed to weigh in a larger sample size (2.5 g freeze-dried). The extractions were performed in separation funnels as opposed to small tubes in **Paper I** because of a larger extraction volume. This significantly reduced the throughput of the method, and the analysis was therefore only performed in duplicate. The clean-up steps were SPE and semi-preparative HPLC using a fraction collector to collect vitamin D_2 and ergosterol separately (**Paper II**). The detection method used was essentially as described in **Paper I**. Examples of chromatograms for a typical grass sample can be seen in Figure 6.2.

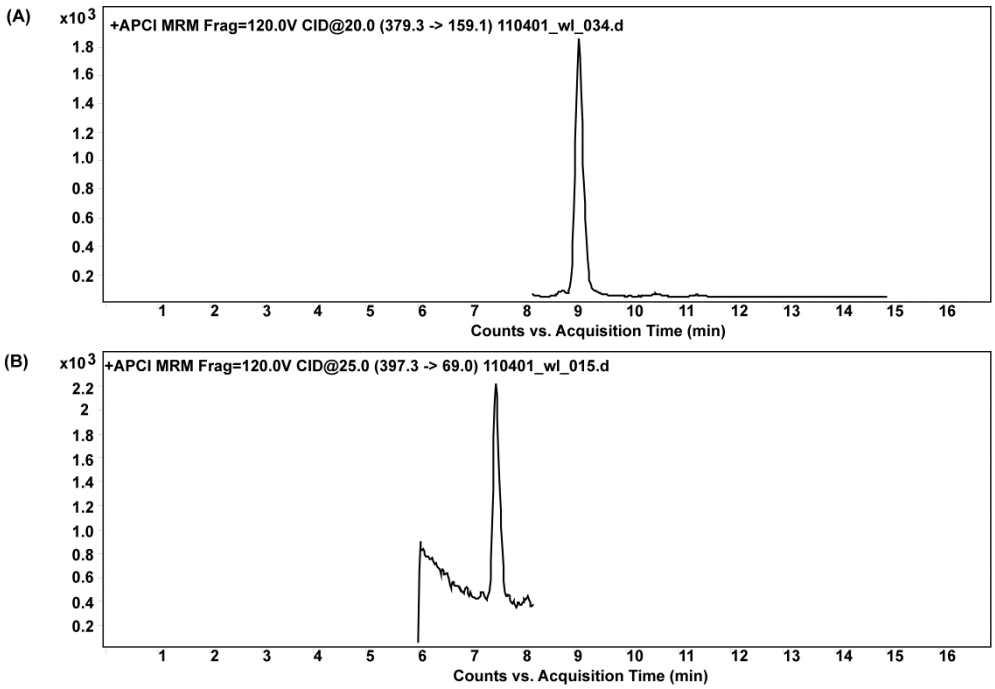

Figure 6.2. Chromatograms for quantifier transitions of (A) ergosterol and (B) vitamin D_2.

The content of ergosterol and vitamin D_2 was analyzed in six varieties of perennial ryegrass harvested four times during the season 2010 (**Paper II**). The content of ergosterol and vitamin D_2 changed more than a factor of ten during the season. The average content of vitamin D_2

found was 2 µg/kg (**Paper II**). The vitamin D_2 content in the perennial ryegrass analyzed in the present study was maximum 2‰ of the ergosterol content. This suggests ergosterol is not the main limiting factor for vitamin D_2 synthesis and the effect of weather was, therefore, exploited. Sun, temperature and precipitation were recorded and a principal component analysis was performed to study the influence on the formation of ergosterol and vitamin D_2. This suggested that a combination of weather factors is involved in the formation of vitamin D_2. Precipitation and high humidity are essential for ergosterol synthesis, whereas sun is necessary for vitamin D_2 synthesis. A combination of precipitation and sun are, therefore, optimal for production of vitamin D_2 in perennial ryegrass.

Only two previous studies have used selective chemical methods to determine the content of vitamin D_2 in plants (Horst et al., 1984;Magalhães et al., 2007). Horst et al. (1984) examined sun-cured field grown alfalfa (*Medicago sativa* L.) and found 48 µg vitamin D_2/kg. If we adjust for differences in dry matter between hay and fresh grass is this almost similar to the contents presented in **Paper II**. Magalhães et al. (2007) studied different varieties of hop (*Humulus lupulus* L.) and found vitamin D_2 and ergosterol in only one variety. Compared to the present study was the ergosterol content (1840 µg/kg dry wt.) at the same level, but the vitamin D_2 content significantly higher (1950 µg/kg dry wt.). One explanation for the higher content of vitamin D_2 in hop could be a difference in UVB-exposure. The hop was dried outside in Portugal during the summer, whereas the grass in the present study was analyzed directly after harvest in Denmark. Portugal is situated at lower latitude than Denmark, which also could explain a difference in the vitamin D_2 content.

6.2 Occurrence of vitamin D_3 related compounds in plants

The synthesis of vitamin D_3 in plants is unresolved and contradicting results concerning the dependence of UVB-exposure have been presented. Two studies (**Paper I** and **Paper III**) on vitamin D_3 synthesis and metabolism in various plants were, therefore, conducted. The main results from these two studies will be discussed in this section. The first study, described in **Paper I**, was intended as a pilot study. The analyzed compounds were vitamin D_3, 7-dehydrocholesterol, cholesterol and cycloartenol. The aim of the second study, described in **Paper III**, was to obtain a better understanding of vitamin D_3 synthesis and metabolism in plants. Vitamin D_3, its hydroxylated metabolites and its glycoside conjugates were analyzed in the second study. Additionally were 7-dehydrocholesterol, cholesterol, lanosterol and major plant sterols analyzed.

6.2.1 Vitamin D_3 and 7-dehydrocholesterol

Vitamin D_3 and 7-dehydrocholesterol were analyzed by LC-APCI-MS/MS in the first study, whereas Diels-Alder derivatization followed by LC-ESI-MS/MS was used for vitamin D_3 in the second study. The results obtained in the two studies are summarized in Table 6.1. The plants analyzed in the first study were *S. glaucophyllum*, *S.lycopersicum*, *S. bicolor*, *C. annuum* and *P. sativum*. These plants were exposed to UVB-light during growth as described in **Paper I**. The plants used for the second study were *S. glaucophyllum, S. lycopersicum* and *C. annuum*. These plants were exposed to UVB-light and heat during growth as described in **Paper III**.

Vitamin D_3 and 7-dehydrocholesterol were identified in leaves of *S. glaucophyllum, S. lycopersicum* and *C. annuum*, which all belong to the taxonomic family *Solanaceae* (**Paper I** and **Paper III**). Either vitamin D_3 or 7-dehydrocholesterol was identified in *P. sativum* and *S. bicolor* belonging to *Fabaceae* and *Poaceae*, respectively (**Paper I**). Limited plant material was in general available and only one determination per plant was, therefore, possible. However, the content of vitamin D_3 in *S. glaucophyllum* and *S. lycopersicum* is almost identical in the two studies. The 7-dehydrocholesterol level reported for *S. glaucophyllum* was on the other hand considerably higher in the second study, which cannot be explained.

Table 6.1. Content of 7-dehydrocholesterol and vitamin D_3 (µg/g dry wt.) in *S. glaucophyllum*, *S. lycopersicum*, *S. bicolor*, *C. annuum* and *P. sativum*. The plants were either grown at elevated temperatures (Heat), UVB treated during growth (UVB) or a combination (Heat + UVB)

Plants	Study	Treatment	7-dehydrocholesterol	Vitamin D_3
S. glaucophyllum	Paper I	Control	0.67	-
		UVB	1.26	0.21
	Paper III	Control	23	0.0032
		UVB	1.6	0.20
		Heat	16	0.0055
		Heat + UVB	2.7	0.10
S. lycopersicum.	Paper I	Control	0.47	-
		UVB	0.23	0.10
	Paper III	Control	0.40	0.0017
		UVB	0.09	0.10
		Heat	0.59	0.0023
		Heat + UVB	0.17	0.11
C. annuum	Paper I	Control	0.03	-
		UVB	0.03	-
	Paper III	Control	0.17	-
		UVB	-	0.0029
		Heat	0.33	-
		Heat + UVB	0.09	0.0063
P. sativum	Paper I	Control	-	-
		UVB	-	-
S. bicolor	Paper I	Control	-	-
		UVB	-	-

("-" = not identified)

The 7-dehydrocholesterol level in *S. glaucophyllum* and *S. lycopersicum* is similar to what has been found elsewhere (Björn and Wang, 2001;Aburjai et al., 1996). No comparable data exist for either 7-dehydrocholesterol or vitamin D_3 in *C. annuum*. The vitamin D_3 content detected in *S. lycopersicum* in previous studies vary from 0.09 µg/g dry wt. to 1.1 µg/g fresh wt. (Aburjai et al., 1998;Prema and Raghuramulu, 1996;Björn and Wang, 2001). The difference

between the studies could be due to the growth conditions used, e.g., the intensity of the light and the length of exposure. The content of vitamin D_3 in *S. glaucophyllum* has only been quantified in cells, which was found to contain vitamin D_3 between 2.2-42.1 µg/g dry wt. (Aburjai et al., 1996). This is significantly higher than the present study. However, the difference in vitamin D_3 content observed between *in vitro* and *in vivo* conditions are in accordance with other studies and may be explained by transformations occurring in the culture medium (Curino et al., 2001).

The isomerization of previtamin D_3 to vitamin D_3 is a temperature-dependent reaction and an effect of growth temperature could therefore be expected, but no consistent effect was seen (Table 6.1). Vitamin D_3 has in most studies been identified after UVB-exposure (Zucker et al., 1980;Björn and Wang, 2001;Aburjai et al., 1996) and this was also the case in the first study (**Paper I**). However, in the second study was vitamin D_3 identified in both UVB- and non-UVB-treated plants (**Paper III**). This is in accordance with a previous study were vitamin D_3 were identified in *S. glaucophyllum* cultured under strict conditions of darkness (Curino et al., 1998). The content of vitamin D_3 in the UVB-treated plants was 18-64 times higher than for the non-UVB-treated plants (Table 6.1). Failure to detect vitamin D_3 in non-UVB-treated plants in previous studies could be due to the use of relative insensitive assays.

6.2.2 Sterols

Sterols function as regulators of membrane fluidity and permeability, and act as precursors of steroids including vitamin D_3. A correlation between the general sterol level and the content of vitamin D_3 in plants could, therefore, be expected. Cholesterol and major plant sterols were therefore analyzed. The sterols analyzed in the first study were cholesterol and cycloartenol, whereas cholesterol, cycloartenol, lanosterol, sitosterol, campesterol and stigmasterol were analyzed in the second study. The results are summarized in Table 6.2.

Lanosterol, which is the precursor for cholesterol in vertebrates and fungi, were not found in any of the plants (**Paper III**). Cholesterol accounted for 9-12% of the quantified total sterols, which is similar to other findings in *Solanaceae* (Zygadlo, 1993). The highest sterol content was in general found in samples treated with both UVB and heat (Table 6.2;**Paper III**). UVB and heat stress the plants, which induce a variety of biochemical changes. Especially, the

ability of membranes to maintain fluidity within an optimum range will help determine its resistance and adaption to stress (Lurie et al., 1995;Berli et al., 2010). The fluidity is a consequence of interactions between sterols and phospholipids and heat gives an increase in the sterol level with a concomitant increase in the sterol:phospholipid ratio (Lurie et al., 1995; Hamada, 2001). However, no correlation could be observed between a high sterol content and a high vitamin D_3 content, but this needs further investigation as the data material was limited.

Table 6.2. The occurrence of cholesterol, cycloartenol, sitosterol, campesterol and stigmasterol in *S. glaucohyllum*, *S. lycopersicum*, and *C. annuum* (µg/g dry wt.). The plants were either grown at elevated temperature (Heat), UVB treated during growth (UVB) or a combination (Heat + UVB)

	Treatment	***Study***	***S. glaucophyllum***	***S. lycopersicum***	***C. annuum***
Cholesterol	UVB	Paper I	68	45	7.1
	Control		60	56	8.3
	UVB	Paper III	30	1.3	3.0
	Heat		67	6.9	3.4
	Heat + UVB		82	18	2.9
	Control		67	10	1.8
Cycloartenol	UVB	Paper I	57	-*	8
	Control		34	-*	15
	UVB	Paper III	82	91	54
	Heat		185	41	48
	Heat + UVB		135	242	81
	Control		144	29	24
Sitosterol	UVB	Paper III	173	7.2	5.2
	Heat		220	23	5.1
	Heat + UVB		347	12	3.2
	Control		265	6.3	3.6
Campesterol	UVB	Paper III	27	5.7	2.1
	Heat		45	5.6	2.3
	Heat + UVB		146	1.0	1.4
	Control		42	4.5	2.1
Stigmasterol	UVB	Paper III	29	28	2.3
	Heat		32	22	3.8
	Heat + UVB		50	40	2.2
	Control		32	18	2.5

(-)* = interferences present, which made it impossible to quantify cycloartenol

6.2.3 Hydroxylated metabolites of vitamin D_3

The occurrence of $25OHD_3$ and $1,25(OH)_2D_3$ were investigated in the leaves of *S. glaucophyllum, S. lycopersicum* and *C. annuum* using LC-ESI-MS/MS in combination with Diels-Alder derivatization. This revealed the presence of $25OHD_3$ in *S. glaucophyllum, S. lycopersicum* and *C. annuum* (**Paper III**). All samples of *S. glaucophyllum* contained $25OHD_3$, but the content in the UVB-treated plants was 14-39 times higher than for non-UVB-treated plants. Only UVB-treated *S. lycopersicum* and *C. annuum* contained $25OHD_3$. The dihydroxylated metabolite ($1,25(OH)_2D_3$) was only identified in UVB-treated *S. glaucophyllum* (**Paper III**). It may also be present in *S. lycopersicum* and *C. annuum*, but below LOD (0.1 ng/g dry wt.). Prema and Raghramulu (1996) identified, contrary to the present study, 100 ng/g dry wt. of $1,25(OH)_2D_3$ in *S. lycopersicum*, but this value was assessed by a less selective UV detection method. A significantly higher content has also been reported for $25OHD_3$ and $1,25(OH)_3D_3$ in *S. glaucophyllum* cell cultures (Aburjai et al., 1996). Synthesis of $1,25(OH)_2D_3$ has been shown to be greatly influenced by culture conditions and especially availability of calcium, which may explain this difference (Curino et al., 2001).

The ratio between vitamin D_3 and its hydroxylated metabolites provides important information about the biosynthesis and regulation of the enzymes involved. The content of $25OHD_3$ in *S. lycopersicum* is 22-28 times lower than for vitamin D_3, whereas it is 6-16 lower for *C. annuum* (**Paper III**). Similar has the vitamin $D_3/25OHD_3$ ratio previously been determined to 7 for *S. lycopersicum* (Aburjai et al., 1998). The content of $25OHD_3$ in *S. glaucophyllum* is 6-9 times lower than for vitamin D_3, whereas the concentration of $25OHD_3$ and $1,25(OH)_2D_3$ is equal (**Paper III**). This indicates that vitamin D_3 is synthesized first and afterwards transformed into $25OHD_3$ and that conversion of $25OHD_3$ to $1,25(OH)_2D_3$ not is as tightly regulated as in vertebrates.

6.2.4 Vitamin D_3 glycosides

Enzymatic hydrolysis was used to study the occurrence of glycoside conjugates. These were found exclusively for $1,25(OH)_2D_3$ in UVB-treated *S. glaucophyllum* (17 ng/g dry wt.) (**Paper III**). The glycoside content has been suggested to increase by improper drying and storage of plant material (Prema and Raghuramulu, 1994; Peterlik et al., 1977). The plant

material used in this study was freeze-dried and stored at -80°C until analysis, which should diminish these effects. Sterol glycosyltransferases in plants catalyze glycosylation of sterols and related compounds and high temperatures can activate glucosyltransferases (Madina et al., 2007). However, the temperature effect on the glycoside content was not investigated in the present study.

The specific site of glycosylation, the type of glycosidic bond involved and the identity of the carbohydrate unit is not completely determined. All three hydroxyl groups in $1,25(OH)_2D_3$ could be sites for glycosylation, but position 3 is the most likely (Grille et al., 2010). The number of glycoside units or the identity seems to differ, since the glycosides from *S. glaucophyllum* and *Trisetum flavescens* are soluble in water (Wasserman et al., 1976b;Napoli et al., 1977;Morris and Levack, 1982), while the glycoside of *Cestrum diurnum* is less polar and soluble in a mixture of chloroform and methanol (Wasserman et al., 1976a;Hughes et al., 1977). Vidal et al. (1985) isolated the $1,25(OH)_2D_3$ glycoside from *S. glaucophyllum* and found that $1,25(OH)_2D_3$ was bound to a series of fructoglucosides. The structure was investigated through periodic acid oxidation, which demonstrated that the fructose was linked to a disaccharide unit repeating one, two or four times (Vidal et al., 1985).

The formation of glycosides may cause dramatic changes in the chemical, nutritional and metabolic properties of vitamin D_3 and its metabolites. Rambeck et al. (1984) studied the biological activity of mono-glycosides of $1\alpha(OH)D_3$ and vitamin D_3 and the corresponding parent molecules in bioassays using rats, chickens and quails. In rats and chickens, vitamin D_3 and the vitamin D_3 β-D-glucoside exhibited nearly equivalent activity (Rambeck et al., 1984). In contrast, the β-D-glucoside of $1\alpha(OH)D_3$ exhibited only 10% activity relative to $1\alpha(OH)_2D_3$ in all the bioassays, whereas the disaccharide derivate exhibited no vitamin D activity in the chicken bioassay (Rambeck et al., 1984). No such study have been performed on $25OHD_3$ and $1,25(OH)_2D_3$. Aqueous extracts of *S. glaucophyllum* leaves preincubated with bovine ruminal fluid (De Boland et al., 1978) and ovine ruminal fluid (Esparza et al., 1983) exhibited more vitamin D activity than extracts not incubated. Later the presence of vitamin D_3 and its metabolites were identified in *S. glaucophyllum* extracts incubated with ovine ruminal fluid (Skliar et al., 1992). This indicates that vitamin D_3 and its metabolites may be liberated from its glycosides at least in ruminants.

6.3 Biosynthesis of vitamin D_3

The present PhD thesis confirms that vitamin D_3 can be found in *S. glaucophyllum*, *S. lycopersicum* and *C. annuum*. However, it still needs to be fully established how these compounds are formed. Vitamin D_3 was found in both UVB- and non-UVB-treated plants, but in significantly higher amounts in UVB-treated plants. It is possible that besides an UVB-dependent pathway an alternative minor non-photolytic reaction from 7-dehydrocholesterol to vitamin D_3 takes place in plants. Non-photolytic production of vitamin D_3 has been proposed to take place by enzyme-catalyzed conversion of 7-dehydrocholesterol to vitamin D_3 via retro-ene rearrangement, by enzymatic epoxidation of 7-dehydrocholesterol or through a pathway involving radical intermediates (Norman and Norman, 1993).

The reported 7-dehydrocholesterol content was in general 2-14 times lower after UVB-exposure, whereas the effect of heat-treatment was minimal (0.7-1.9 times difference) (**Paper III**). The lower content of 7-dehydrocholesterol in UVB-treated plants could be related to a conversion to vitamin D_3 after UVB-exposure. However, not all 7 -dehydrocholesterol lost was converted to vitamin D_3, which indicates that vitamin D_3 either is transformed inside the plant e.g., to hydroxylated metabolites or that some previtamin disappear in side reactions. Prolonged UVB-exposure converts previtamin D_3 to the inactive forms lumisterol and tachysterol in vertebrates, protecting the organism from vitamin D toxicity (Holick et al., 1981). If similar side reactions are present in plants remain to be established. It is also likely that some 7-dehydrocholesterol could enter other pathways e.g., be transformed into cholesterol.

The last step in cholesterol biosynthesis in vertebrates is the reduction of the Δ-7 double bond of 7-dehydrocholesterol by the enzyme $\Delta^{5,7}$-sterol-Δ-7-reductase (7DHCR) to give cholesterol (Glossmann, 2010) and similar $\Delta^{5,7}$-sterol-Δ-7-reductases (DWARF5) exist in plants (Schaller, 2003) (Figure 6.3). Many enzymes involved in the biosynthesis of sterols do not have absolute substrate specificity (Benveniste, 1986). It is, therefore, possible that DWARF5 could act on 7-dehydrocholesterol in plants to form cholesterol. The activity of DWARF5 may control the cholesterol/7-dehydrocholesterol ratio. However, it is unknown how 7-dehydrocholesterol and cholesterol is formed in plants in the first place.

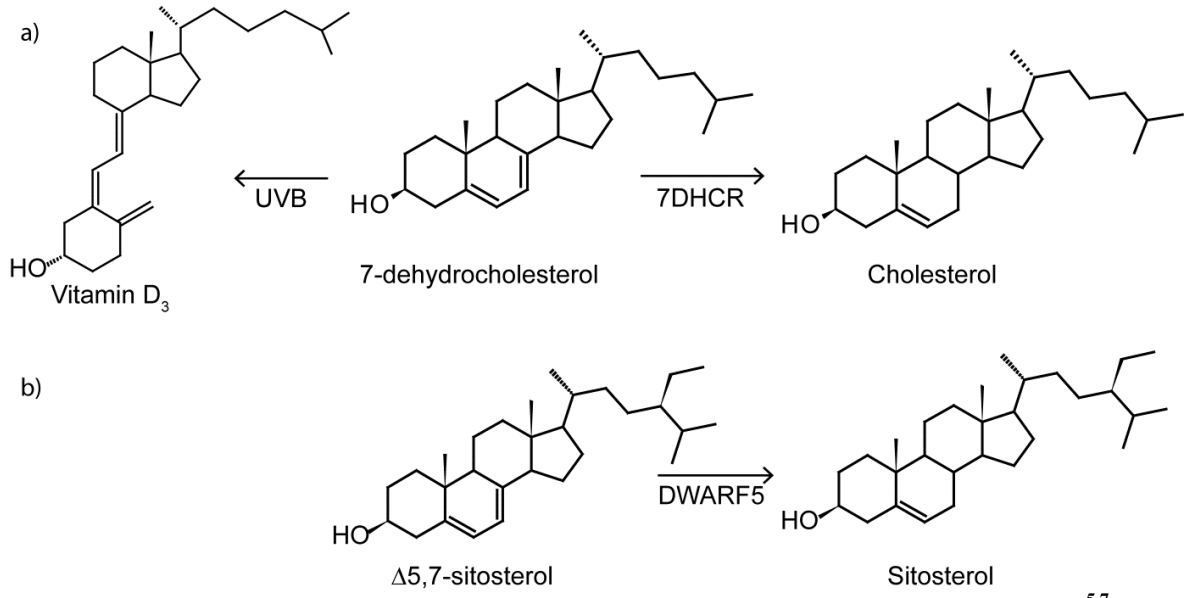

Figure 6.3. a) The conversion of 7-dehydrocholesterol to cholesterol by the action of $\Delta^{5,7}$-sterol-Δ-7-reductase (7DHCR). By UVB-exposure is 7-dehydrocholesterol converted to vitamin D_3 b) The conversion of Δ5,7-sitosterol into sitosterol by the action of DWARF5 (Modified from Paper III).

As mentioned in Section 3.4, plant sterols are synthesized via cycloartenol catalyzed by cycloartenol synthase (CAS) in plants, whereas cholesterol and 7-dehydrocholesterol are synthesized via lanosterol catalyzed by lanosterol synthase (LAS) in vertebrates (Ohyama et al., 2009). Although numerous labelling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor, were putative LAS genes recently identified in *Arabidopsis thaliana* (Ohyama et al., 2009;Suzuki et al., 2006;Kolesnikova et al., 2006), *Panax Ginseng* (Suzuki et al., 2006) and *Lotus japonica* (Kolesnikova et al., 2006;Sawai et al., 2006). This suggests that LAS genes might be widespread among eudicots. Lanosterol may act as an alternative intermediate for the synthesis of sterols, thus cholesterol and 7-dehydrocholesterol may be formed through a pathway similar to the one known from vertebrates. Only cycloartenol was detected in the present study, which does not support this hypothesis. It seems that SMT1, that catalyzes the first methylation of cycloartenol to 24-methylene cycloartenol interfere with the accumulation of cholesterol in plants (Figure 6.4). Cholesterol accumulates in plants bearing a SMT1 null mutation, which indicates that the production of high amounts of cholesterol results from a by-pass of SMT (Diener et al., 2000). This could be a tool for bio-fortification of plants to force the pathway in direction of 7-dehydrocholesterol. However, any increase in accumulation of 7-dehydrocholesterol and by that means vitamin D_3 should be viewed in the context of the overall changes in the metabolic profile e.g., in which the level of other compounds changes.

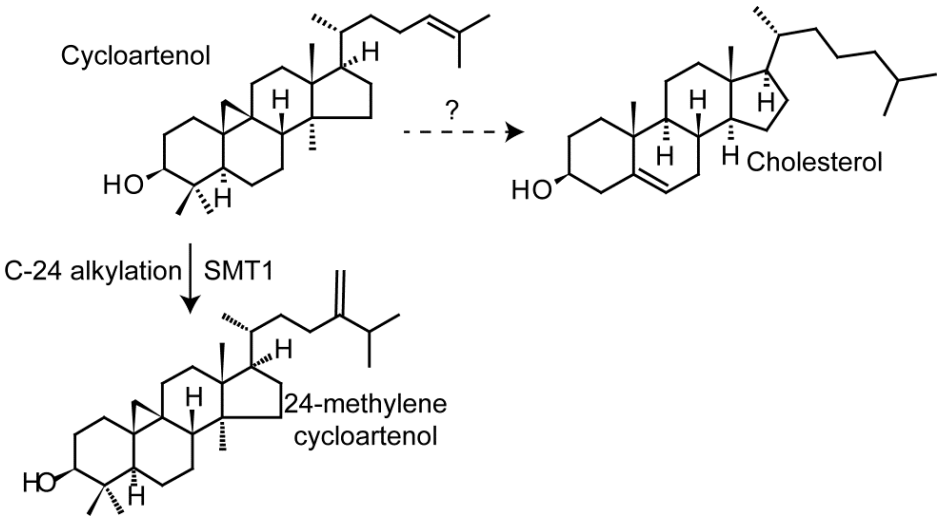

Figure 6.4. Action of sterol methyltransferase 1 (SMT1) on cycloartenol to yield 24-methylene cycloartenol. It is unknown how cholesterol is formed, but studies indicate that cholesterol accumulates in plants bearing a SMT1 null mutation (Paper III).

It remains to be discovered whether vitamin D_3 in plants is formed from lanosterol and/or cycloartenol in plants and labelling experiments with cycloartenol and lanosterol is needed to determine the origin of cholesterol and 7-dehydrocholesterol in plants.

7 Conclusion

The primary aim of this thesis was to study the occurrence and biosynthesis of vitamin D in plants, with the scope of testing the hypothesis that plants can be a source of vitamin D for humans as well as for animals. Sensitive and selective analytical methods are a prerequisite to obtain trustworthy data and several methods were, therefore, developed to study vitamin D, its sterol precursors and its hydroxylated metabolites in more details. All methods were based on liquid chromatography combined with mass spectrometry detection because of the superior selectivity and sensitivity. The optimum detection principle was found to be tandem mass spectrometry using a triple quadrupole instrument, which proved to be useful to evaluate the content of vitamin D in plants. However, the original goal to develop a high throughput method was not achieved as more than one sample preparation step had to be included. The developed methods were used in three studies covering various aspects of vitamin D_2 and vitamin D_3 in plants. The following conclusions can be drawn on the basis of these studies:

Occurrence and seasonal variation of vitamin D_2 and ergosterol in grass

- The average content of vitamin D_2 in perennial ryegrass was 2 μg/kg, which is lower than expected from previous studies using bioassays.
- The vitamin D_2 content was maximum 2‰ of the ergosterol content.
- The content of both ergosterol and vitamin D_2 is changing more than a factor of ten during the season.
- A combination of sun and precipitation is important for the formation of vitamin D_2.

Vitamin D_3 and its sterol precursors

- Vitamin D_3 was identified in the leaves of *S. glaucophyllum, S. lycopersicum, C. annuum,* belonging to the taxonomic family *Solanaceae.*
- Vitamin D_3 was identified in both UVB- and non UVB-treated plants, but the content of the UVB-treated plants was 18-64 times higher.
- No vitamin D_3 was identified in either *P. sativum* or *S. bicolor* belonging to *Fabaceae* and *Poaceae*, respectively.

- Both cholesterol and 7-dehydrocholesterol were found in all vitamin D_3 synthesizing plants and may serve as precursors for vitamin D_3 in plants.

Hydroxylated metabolites and glycosylated forms of vitamin D_3

- $25OHD_3$ was identified in the leaves of *S. lycopersicum*, *C. annuum* and *S. glaucophyllum*, whereas $1,25(OH)_2D_3$ only was identified in the leaves of *S. glaucophyllum*.
- Enzymatic hydrolysis was used to study the occurrence of glycoside conjugates. These were found exclusively for $1,25(OH)_2D_3$ in UVB-treated *S. glaucophyllum.*

8 Perspectives

The hypothesis, which is the background for the present PhD thesis, is that plants can be a source of vitamin D for humans as well as for animals. The conclusion, based on this PhD thesis, is that both vitamin D_2 and vitamin D_3 can be found in plants. However, plants have to contribute significantly to the recommended intake of vitamin D to be considered as a vitamin D source. Considerations and future perspectives for plants as a vitamin D source are given in this chapter.

8.1 Grass a source of vitamin D_2

Although the content of vitamin D_2 in grass is low, it has to be taken into account that e.g., a lactating cow, as a rule of thumb, eat dry matter equivalent to 3.2 percent of their body-weight each day (NRC, 1987). If the weight of the cow is 700 kg, the intake will be 22.4 kg dry matter, which corresponds to 23 kg hay and 117 kg of the material studied. This corresponds to an intake of 8-747 µg vitamin D_2 per day if the cow only were fed the grass analyzed in the present study. The National Research Council (NRC) recommends that a lactating cow is provided with 30 IU/kg vitamin D per day that is 21000 IU or 525 µg for a 700 kg cow (NRC, 2001). This demonstrates that grass potentially can be a significant source of vitamin D for grazing animals and animals fed on silage and hay. It is in that connection valuable to study the effect of various curing methods on the vitamin D_2 content. Exposure of grass or hay to sunlight or artificial UVB-light during curing would probably increase the content of vitamin D_2, which would be a benefit especially during winter when the animals not have access to outdoor sunlight. Higher vitamin D content in animal feed would later be a benefit for humans owing to a higher content of vitamin D in the produced milk and meat. However, Hymøller and Jensen (2010) found significantly higher levels of vitamin D_3 than vitamin D_2 in cows after ingestion of equal amounts of the two vitamins. It needs to be investigated if vitamin D_2 and vitamin D_3 have equal activity to fully evaluate grass as a source of vitamin D. Vitamin D_2 in grass is a benefit, but vitamin D_2 in grass is also linked to a risk aspect. High vitamin D_2 is associated with a high content of ergosterol, which is a measure of fungal growth. Fungal growth may lead to the formation of mycotoxins, which may cause diseases if consumed by animals (Scudamore and Livesey, 1998). Thus, a high content of vitamin D_2 in grass may be at the expense of the feeding quality. Further investigations are needed to study the correlation between mycotoxins, ergosterol and vitamin D_2.

8.2 Plants as a source of vitamin D_3

Vitamin D deficiency is a problem in populations with limited sun exposure and dietary intake of vitamin D becomes essential. However, very few food sources naturally contain vitamin D and the general population as a result fail to meet their vitamin D requirements. Fortification could be part of the solution, but will not meet the requirements in all groups. It would therefore be valuable to increase the sources of vitamin D_3 in the human diet, and to optimize the content by biofortification. Traditionally has only animal products been considered a source of vitamin D_3, but the present study confirms that vitamin D_3 also exists in plants. People with a low intake of animal products typically have a high intake of fruit and vegetables making plants a good new source of vitamin D. It seems that especially the *Solanaceous* family contain high amounts of vitamin D_3. This is of particular interest because the *Solanaceous* family includes potato and tomato and other important vegetables consumed by humans. It is of interest to determine the distribution of vitamin D_3 compounds in various species at different growth conditions and to identify if also the fruits contain vitamin D_3 to evaluate the potential of plants as a new source of vitamin D. However, to carry out a larger screening of vitamin D_3 in plants it would be necessary to automate some of the sample preparation steps for instance by online SPE to make the methods less time-consuming.

The Danish dietary recommendation for fruit and vegetables is 600 g a day. It could provide us with an additional 0.2-12 µg of vitamin D_3 per day if all 600 g was supplied by the UVB-treated leaves analyzed in the present study. To put it into perspective: 0.2 µg corresponds e.g. to 100 g butter (Jakobsen and Saxholt, 2009), whereas 12 µg corresponds to e.g. 50 g salmon (Chen et al., 2007). Consequently, UVB-exposure of certain plants during growth could be used as a source of bio-fortification to increase the vitamin D_3 content of plants. The calculated intake would be even higher if the hydroxylated metabolites were included in the calculation. However, it is impossible to calculate the contribution of the vitamin D metabolites to the vitamin D activity due to lack of information. The biologically active form, $1,25(OH)_2D_3$, is present at a level only ten times lower than vitamin D_3. The relative contribution to the vitamin D activity is, therefore, expected to be relevant, as the potency of $1,25(OH)_2D_3$ has been attributed to ten relative to vitamin D_3 (Tanaka et al., 1973). Limited data exist on $1,25(OH)_2D_3$ in food in general, this is most likely because of a lack of analytical methods. This thesis demonstrates that sensitive and selective detection of $1,25(OH)_2D_3$ in plants is possible and the challenge from now on will be to optimize the

extraction and sample preparation procedure. Extraction of $1,25(OH)_2D_3$ from the non-saponifiable matter was difficult and low recovery was observed. There is a need for more data on the level of esters or other conjugated forms of $1,25(OH)_2D_3$ in food to choose the best extraction procedure. In regard to plants there is a special interest to investigate the level of glycosides because glycosylation can have a huge impact on the bioavailability. In general, improvement and development of analytical procedures are needed to study vitamin D glycosides in more details. Synthesis of various glycosylated standards would be of special interest to develop an optimal analytical method to study the occurrence of glycoside conjugates in plants.

It still needs to be fully established how vitamin D_3 is formed in plants. Both cholesterol and 7-dehydrocholesterol were found in all vitamin D_3-synthesizing plants and may serve as precursors of vitamin D_3 in plants. However, the biosynthesis of these sterols in plants is still unknown and labelling experiments with cycloartenol and lanosterol are needed to determine the origin. Elucidation of the biosynthetic pathway of vitamin D_3 could provide tools to increase the biosynthesis of vitamin D_3 in plants by molecular breeding. However, sterol biosynthesis in plants is complex and is still poorly understood and makes this an immense challenge.

8.3 Concluding remarks

Overall, this PhD thesis has shown that plants potentially can be a source of both vitamin D_2 and vitamin D_3 for humans as well as for animals. However, it seems that even more questions now can be asked: How do plants synthesize vitamin D_3? Is it a general ability of plants? Is vitamin D_3 also synthesized in the fruits? Which plants species synthesize vitamin D_3? How can we increase the content of vitamin D_2 in grass without a decrease in quality of the feed? I hope that this thesis will be a stepping stone towards an answer and an inspiration for future research that eventually could form the basis for plants with a natural higher content of vitamin D. However, selective and sensitive quantification of vitamin D metabolites in plants by analytical methods that are less time-consuming will be a challenge to reach this goal. The methods developed in this PhD thesis can serve as starting point.

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10 Appendix

Table 10.1. Content of ergosterol (µg/kg fresh wt.) in the preliminary study on grass. RSD% corresponds to relative standard deviations when determinations where made in triplicates

Variety	Ergosterol (µg/kg fresh wt.)
Trifolium repens L. (Klondike)	900, RSD%=3
Trifolium repens L. (Riesling)	730
Trifolium repens L. (Rivendel)	1480
Trifolium pratense L (Amos)	70
Trifolium pratense L. (Rajah)	170
Medicago Sativa L (Daisy)	100
Phleum pratense L. (Dolina)	300
Dactylis glomerata L. (Amba)	470, RSD%=11
Dactylis glomerata L. (Donata)	290
Festuca pratensis Huds. (Jamaica)	625
Festulolium (Hykor)	230
Festulolium (Perun)	145
Festuca arundinacea Schreb. (Jordane)	280
Lolium x boucheanum Kunth (Storm)	250, RSD%=2
Lolium x boucheanum Kunth (Tetratop)	160
Poa pratensis L. (Oxford)	620
Lolium perenne L. (Kimber)	605
Lolium perenne L. (Telstar)	1030
Lolium perenne L. (Calibra)	630
Lolium perenne L. (Indiana)	870. RSD%=8
Lolium perenne L. (Premium)	770
Lolium perenne L. (Turandot)	1410
Lolium perenne L. (Asturion)	635
Lolium perenne L. (Cancan)	390
Lolium perenne L. (Foxtrot)	365
Lolium perenne L. (Pastour)	340
Lolium perenne L. (Polim)	170
Lolium perenne L. (Tivoli)	255
Phleum pratense L. (Winnetou)	600
Festuca arundinacea Schreb. (Kora)	555

11 Publications

Paper I

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Analytical Methods

LC–MS/MS with atmospheric pressure chemical ionisation to study the effect of UV treatment on the formation of vitamin D_3 and sterols in plants

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UV treatment

ABSTRACT

Some plant species are known to cause calcium intoxification in grazing animals. This has been attributed to the presence of vitamin D_3-like activity. However, research into the presence of vitamin D_3 in plants has been limited. One reason for this may be limitations in the analytical methods available for unambiguous detection and quantification of vitamin D_3. This paper presents a new method for determining vitamin D_3 and its sterol precursors. The method is based on saponification and extraction followed by solid phase clean-up of the compounds from plant leaves and detection by APCI-MS. Recoveries ranged from 101% to 114% and precision from 3% to 12%. Detection limits were 2–8 ng/g fresh weight for the substances tested. In a pilot study we found that *Solanum glaucohyllum* Desf. and *Solanum lycopersicum* L. produced vitamin D_3 after UV-treatment. The preliminary results presented suggest that vitamin D_3 formation in plants is dependent on light exposure.

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1. Introduction

Some plant species are known to cause calcium intoxification in grazing animals; this has been attributed to the presence of vitamin D-like activity in these plants (Mello, 2003). The term vitamin D includes both vitamin D_2 and vitamin D_3. Vitamin D_2 is produced when ergosterol in fungi and yeast is exposed to UV light, whereas vitamin D_3 is produced in animals by the action of UV radiation on 7-dehydrocholesterol. Vitamin D_3 is usually referred to as animal vitamin D. However, this could be a misconception, because vitamin D_3 and its hydroxylated metabolites have been found to exist in several plant species (Aburjai, Al-Khalil, & Abuirjeie, 1998; Aburjai, Bernasconi, Manzocchi, & Pelizzoni, 1996; Boland, Skliar, Curino, & Milanesi, 2003; Curino, Skliar, & Boland, 1998; Horst, Reinhardt, Russell, & Napoli, 1984; Peterlik, Regal, & Kohler, 1977; Suardi, Bernasconi, Pelizzoni, & Racchi, 1994).

The main function of vitamin D in mammals is the maintenance and regulation of calcium homeostasis and vitamin D deficiency causes rickets in children and osteomalacy in the elderly. Furthermore, vitamin D deficiency has been associated with an increased risk of cancer, cardiovascular diseases, diabetes and reduced immune response (Holick & Chen, 2008). Insufficient vitamin D status is a general issue worldwide; this is probably due to a lack of sun exposure, which is the main source of vitamin D for humans during summer (Holick & Chen, 2008). The dietary intake of vitamin D is small and it will therefore be valuable to find alternative sources of vitamin D including plants. Increase in our knowledge about vitamin D in plants will be of great value as a source for bio-fortification of foods to improve the vitamin D status of the general population. To increase the content of vitamin D in food crops, it is important to obtain a better understanding of how vitamin D synthesis takes place in plants.

Plant sterols, such as sitosterol, are synthesised via cycloartenol in higher plant catalysed by cycloartenol synthase (CAS) (Pathway A, Fig. 1) (Ohyama, Suzuki, Kikuchi, Saito, & Muranaka, 2009). These sterols are precursors of the brassinosteroids, which are plant growth hormones. Ergosterol and cholesterol are both biosynthesised via lanosterol catalysed by lanosterol synthase (LAS) (Pathway B, Fig. 1), in yeast and in mammals (Ohyama et al., 2009).

Despite this, several plant species actually contain cholesterol; especially the *Solanaceae* family contains relatively high amounts of cholesterol (Moreau, Whitaker, & Hicks, 2002). It seems that sterol methyltransferase 1 (SMT1), that catalyses the first methylation of cycloartenol to 24-methylene cycloartenol interfere with the accumulation of cholesterol in plants. (Diener et al., 2000) demonstrated that in *Arabidopsis* plants bearing a SMT1 null mutation, cholesterol was the major sterol, composing 26% of total sterols, compared with 6% in wild-type plants. The cholesterol may be formed by pathway C (Fig. 1). As outlined in Fig. 1 the presence of cholesterol raises the possibility that 7-dehydrocholesterol could be formed in these plants and hence vitamin D_3. However,

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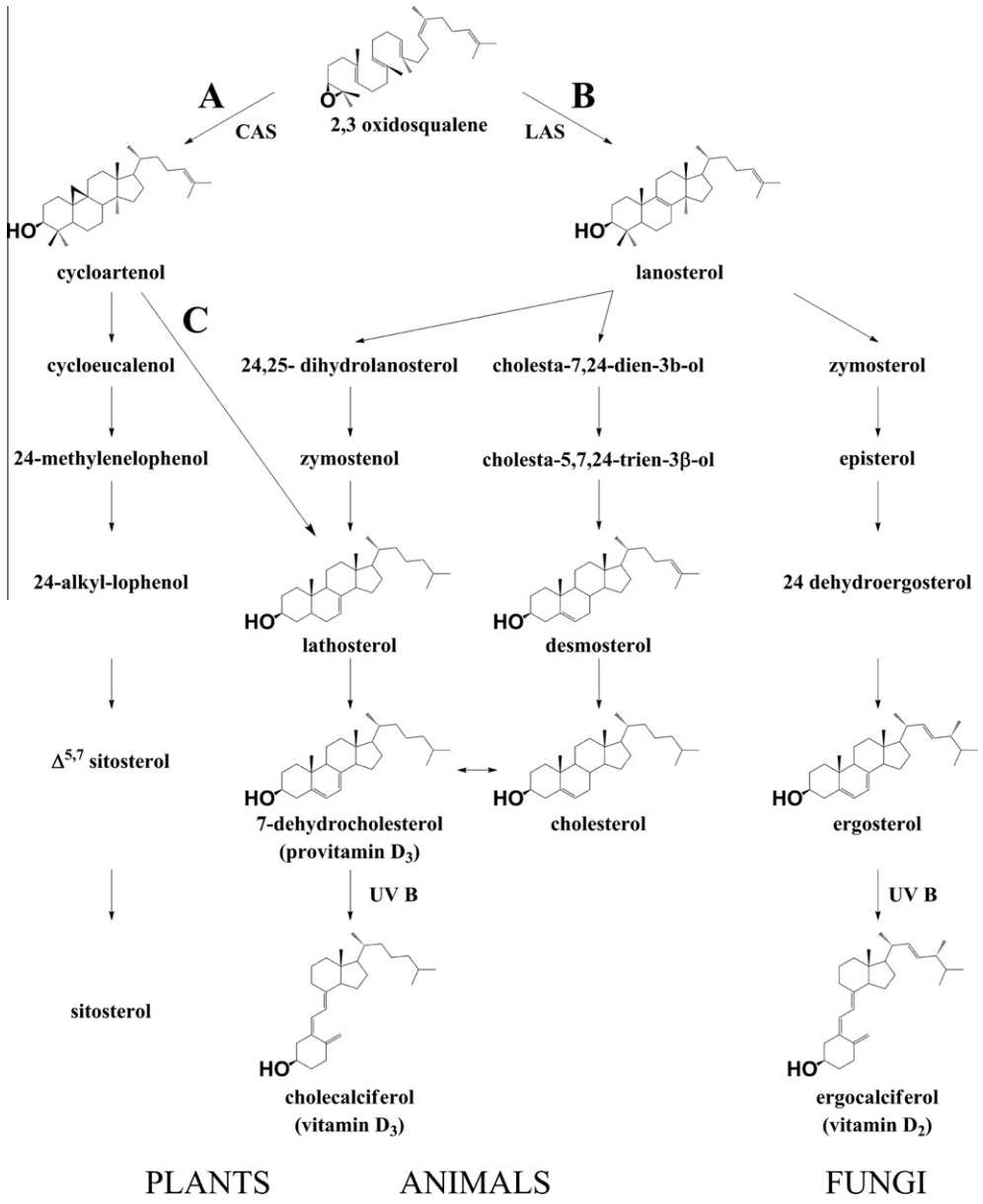

Fig. 1. Sterol biosynthetic pathway through (A) Cycloartenol in Plant and (B) Lanosterol in Animals and Fungi (C) Hypothetical Vitamin D biosynthetic pathway in plant. LAS = Lanosterol synthase, CAS = Cycloartenol synthase.

research into the presence and regulation of vitamin D_3 and 7-dehydrocholesterol in plants has been extremely limited. One reason for this may be the limitations in the analytical methods available for quantifying the specific vitamin D_3 compounds and their sterol precursors.

Sterols are typically measured by gas chromatography (Lagarda, García-Llatas, & Farré, 2006), but this require a time-consuming derivatisation procedure. Furthermore, derivatisation may lead to reduced recovery and the formation of artifacts (Razzazi-Fazeli, Kleineisen, & Luf, 2000). Traditionally, vitamin D_3 has been detected in plants by *in vivo* and *in vitro* bioassays (Boland et al., 2003). Classical bioassays measuring antirachitic activity have long been used to determine vitamin D activity in biological materials (Parrish & Richter, 1979). However, these methods are both time-consuming and imprecise, and cannot distinguish between the different forms of vitamin D. HPLC methods offer the advantage of being able to separate and detect vitamin D. Moreover, HPLC methods followed by UV detection with a diode array detector (DAD) are highly reproducible, but they are quite laborious as they need a high degree of purification, and the sample throughput is low, which makes the cost of the analysis high. Analysis of vitamin D and sterols in complex matrixes such as plant tissue can be especially challenging. However, the problem of the incomplete resolution of compounds in complex samples can be overcome by coupling chromatographic separation with mass spectrometry (MS). The use of LC–MS or LC–MS/MS makes it feasible to investigate vitamin D, its precursors and its metabolites in much more detail and with less sample preparation even in complex biological samples. However, the analysis of sterols and neutral steroids by electrospray ionisation (ESI) is technically challenging, because these compounds lack ionizable groups. Atmospheric pressure chemical ionisation (APCI) has generally been found to be more effective (Martínez-Vidal, Garrido-Frenich, Escobar-García, & Romero-González, 2007).

In the present study, we developed and validated a sensitive method for the analysis of vitamin D and sterols in plants.

Furthermore, the method was applied for determination of 7-dehydrocholesterol, vitamin D_3, cholesterol and cycloartenol in green leaves treated with and without UV.

2. Materials and methods

2.1. Reagents and standards

Methanol, ethyl acetate, pentane and 2-propanol were HPLC-grade (Rathburn Chemicals Ltd., Walkerburn, Scotland). Formic acid and acetonitrile for mass spectrometry was from Fluka (Steinheim, Germany). Ethanol 96% vol was PhEur quality (Kemetyl, Køge, Denmark). L-ascorbic acid was reagent grade (crystalline 20–200 mesh) from Sigma (Steinheim, Germany). Chloroform (PhEur grade), *n*-heptane for liquid chromatography (LiChrosolv) and potassium hydroxide pellets (for analysis) were from Merck (Damstadt, Germany). Hydrochloric acid 1 mol/L was from Bie & Berntsen (Rødovre, Denmark). For preparation of eluents Milli-Q water was used (18 MΩ, Millipore, Billerica, USA).

Various sterol standards were purchased: Lanosterol (97% purity), cholesterol (Purity 95%) cycloartenol (Purity > 90%), vitamin D_2 (Sigma–Aldrich Reference standard), vitamin D_3 (Sigma–Aldrich Reference standard) and lathosterol were from Sigma–Aldrich (Steinheim, Germany). Ergosterol (95%) was from Fluka (Steinheim, Germany). Desmosterol (purity 90%) was from Larodan Fine Chemicals (Malmö, Sweden). 7-dehydrocholesterol (Purity > 98%) was from BioChemika (Steinheim, Germany). Vitamin D_3-[$^2H_3$] was from Isociences (King of Prussia, PA, USA). Standard stock solutions of vitamin D_3, vitamin D_2 and vitamin D_3-[$^2H_3$] were prepared in *n*-heptane. Concentrations of stock solutions of vitamin D_3, vitamin D_2 and vitamin D_3-[$^2H_3$] were assessed by measuring the UV-absorption at 265 nm of dilutions in ethanol. The molar absorption coefficients (ε) in ethanol used for vitamin D_3 and vitamin D_2 were 18,466 and 18,843 respectively (The Pharmaceutical Codex, 1979). Individual standard stock solutions of the sterols were prepared by dissolving the solid compounds in chloroform. The concentration of the final stock solution was calculated taking into account the purity of the commercial standards. The reproducibility of the sterol standards were controlled by preparing the standards twice complete from weighing to calibration curve and comparing the results. The solutions were kept at −20 °C until analysis. Working standard solutions were prepared from these solutions and diluted with methanol prior to analysis.

2.2. Samples

Samples of spinach leaves (*Spinacia oleracea* L.) bought at a local grocery were used as a reference material. UV- and non UV- treated *Solanum glaucophyllum* Desf., *Solanum lycopersicum* L., *Sorghum bicolor* (L.) Moench, *Capsicum annuum* L. and *Pisum sativum* L. were grown in growth chambers with a light/dark cycle of 16/8 (light from 4:00 to 20:00), a temperature setting of 24/17 °C respectively and the light set to 150 μE/(m^2s). Plants of 4–6 true leaves were used for treatments. The UV-B treatment was performed with a bench lamp (UVP-302–15) with 302 nm emission length equipped with 2 × 15 W tubes. Plants were exposed to UV light (in addition to normal light supplied according to the light/dark cycle reported above) for 30 min per day for 7 days (from 6:00 to 6:30). The intensity of the lamp was not measured. The distance from the lamp was set at 1 m from the base of the pot (approx 70 cm from the top of the plant) according to the running method. At the end of the treatment the plant tissues were collected and immediately frozen in liquid nitrogen and freeze-dried (Christ Beta 1–8, SciQuip Ltd, Shropshire, UK). The tissues were finally crushed and homogenised well in a blender (Osterizer, Struer, Denmark). The homogenised samples were stored at −20 °C under a nitrogen atmosphere until analysis.

2.3. Extraction method

The freeze-dried plant material (0.4 g ± 0.1 g) was weight into a 50 mL glass tube with a Teflon-covered lid and mixed with 2 mL 60% potassium hydroxide, 10 mL 96% ethanol and 6 mL 15% ascorbic acid. 250 μL vitamin D_3-[$^2H_3$] internal standard (4 μg/mL in n-heptane) was added to each tube. The tubes were shaken overnight at room temperature (approximately 18 h) in a vibrating tube shaker (Multi Reax, Heidolph GmbH & Co.KG, Schwaback, Germany). Subsequently 15 mL 20% ethyl acetate in pentane (v/v) was added and the tubes were shaken for 30 min in an automatic shaker. The tubes were subsequent centrifuged at 2000*g* for 5 min (Varifuge RF, Heraeus Sepatech, Germany) at room temperature, and the organic layer was withdrawn. This was repeated once. The combined extracts were washed with 20 mL of 0.05 mol/L hydrochloric acid by turning the flask gently 30 times to be sure that the water was free from alkali; this was confirmed with a pH strip. The upper layer was transferred to a round bottom flask and evaporated to dryness in a rotary evaporator at 30 °C (Laborata 4000, Heidolph GmbH & Co.KG, Schwaback, Germany). The residue was redissolved in 2.5 mL 1% 2-propanol in *n*-heptane (v/v) for solid phase clean-up.

2.4. Solid phase extraction

Prior to the application on the analytical column the samples were cleaned-up by solid phase extraction on a silica column (500 mg, 6 mL reservoir, Isolute, IST, Mid Glamorgan, UK) using a vacuum manifold (Vacmaster, IST, Mid Glamorgan, UK). These columns were activated with 5 mL *n*-heptane before the sample extract was loaded. After washing twice with 5 mL 0.5% 2-propanol in *n*-heptane (v/v), the bound compounds were eluted with two aliquot of 4 mL 6% 2-propanol in *n*-heptane (v/v). The eluate was evaporated to dryness under nitrogen (TurboVap, Caliper Life Science, Hopkinton, MA). The residue was finally redissolved in 1.5 mL methanol for analysis. All extracts were filtered through a 0.2 μm filter (Ultrafree-CL 0.2, Millipore, Billerica, MA, USA) prior to injection. Sample extracts were stored at −80 °C until analysis.

2.5. Separation

The liquid chromatographic analysis was done on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) equipped with a thermostated column compartment. The chromatographic separation was done on a Phenomenex Kinetex® PFP column (100 × 2.1 mm, 2.6 μm) fitted with a Phenomenex KrudKatcher ultra column in-line filter (Phenomenex, Torrance, CA) at a flow-rate of 0.3 mL/min. The eluent A was 0.5% formic acid and 25% acetonitrile in Milli-Q water (v/v) and the eluent B was 100% methanol. The gradient program was as follows: 70% B for 1 min, a linear gradient to 80% B for 14 min, a linear gradient to 100% B for 1 min, isocratic elution for 2 min and 3 min linear gradient back to 70% B and re-equilibration for 7 min giving a total run time of 28 min. The column was maintained at 30 °C. 5 μL aliquot was injected. Prior to injection, the needle was washed in the flush port with 2-propanol for 10 s. Furthermore, an injection-valve cleaning program was used at the end of the equilibration period in order to minimise carry-over from the previous sample.

2.6. MS/MS analysis

MS/MS analysis was done on an Agilent 6460 series Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA) equipped with an

atmospheric pressure chemical ionisation (APCI) source. Nitrogen was used as a collision gas. Quantification was done in selected reaction monitoring (SRM) mode. This provides the best combination of sensitivity and selectivity gain compared to full scan and single ion monitoring mode (SIM) techniques.

Ergosterol and vitamin D_2 were used to study the effect of the source parameters by direct infusion experiments optimising the best conditions to maximise peak intensity. Starting with default instrument settings: capillary voltage 2800 V, corona current 6 μA, vaporiser temperature 300 °C, gas temperature 350 °C, nebuliser 20 psi (138 kPa) and gas flow 4 L/min, these parameters were then varied one at a time. The optimal operating conditions were found to be: capillary voltage 2500 V, corona current 3 μA, vaporiser temperature 325 °C, gas temperature 300 °C, nebuliser 15 psi (104 kPa) and gas flow 5 L/min. The collision energy was optimised for each reaction and the fragmentor energy was chosen as 120 for all reactions. To obtain an acceptable chromatographic peak statistics (at least 20 data-points across one peak) SRM reactions were divided into five groups and dwell time was optimised. The precursor ions, product ions and retention times for the compounds studied are shown in Table 1.

MassHunter Workstation software (version B.01.04, Agilent Technologies, Santa Clara, CA) was used for instrument control and data acquisition. Identification of analytes was based on the comparison of their retentions times and of relative abundance of the quantifier and qualifier ions ±20%.

2.7. Validation

The concentration levels expected in the plant samples was tested for linearity (10–1500 ng/mL) using a mixture of standards at the following levels; 10, 25, 50, 100, 500, 1000 and 1500 ng/mL. Three independent injections of the standard mixture were performed at each level. A linear regression was performed between the component/internal standard area ratio and the component/internal standard amount ratio. A validation procedure was performed for vitamin D_3, vitamin D_2, ergosterol and 7-dehydrocholesterol. The accuracy of the method was determined by analyzing spiked samples where 0.4 g freeze-dried spinach leaves was spiked at three spiking levels (37.5, 125 and 500 ng/g fresh weight) for each analyte and measuring the recovery. The internal standard was also spiked into the leaves corresponding to 250 ng/g fresh weight and the recovery was measured. The recoveries were calculated by using external standard. The inter-day reproducibility was checked on three different days at the three spiking levels. The selectivity of the method was determined by carrying out the whole procedure with blank samples containing 60% potassium hydroxide, 96% ethanol and 15% ascorbic acid from start. The selectivity samples were used as blank matrix in the determination of LOD. The limit of detection (LOD) was determined from reproducibility samples (37.5 ng/g fresh weight) diluted by blank matrix to a concentration close to the expected LOD. The LOD was defined as a signal-to-noise ratio of 3.

3. Results and discussion

3.1. Sample preparation

Saponification can be used to liberate free forms of vitamin D and sterols bound as esters. Vitamin D will in a solution be in equilibrium with previtamin D in a thermal dependent manner (Buisman, Hanewald, Mulder, Roborgh, & Keuning, 1968). Overnight cold saponification diminishes thermal isomerisation of vitamin D to previtamin D as compared to hot saponification (Buisman et al., 1968). Thus, we chose to use cold saponification at room temperature overnight. Given the stability of vitamin D and sterols towards oxidation, ascorbic acid was used as an antioxidant along with nitrogen-flushing before saponification. Despite an effort to minimise the sample purification steps it was concluded, based on preliminary experiments, that purification of the extracts was needed. This was necessary both to avoid contamination of the analytical column and the analyser by interfering substances e.g. chlorophyll and other lipophilic pigments. Consequently, a silica solid phase clean-up was included. Due to the low solubility of the sterols, we dissolved the sterols in pure methanol instead of the initial mobile phase prior to injection.

3.2. Chromatographic separation

The small differences in the sterols side chain leads to challenges in obtaining an acceptable separation. Mass spectrometry offers an additional level of resolution by differentiating co-eluting sterols by mass. However, the fact that many sterols equal in mass have to be taken into account. The aim was to develop a single gradient method that resolves related isobaric sterols in the plant extract. Various reversed phase columns were tested and a PFP (pentaflurophenyl) column was chosen over conventional C18 and C8 to obtain faster separation using less organic solvent. The reason being that phenyl material is less hydrophobic than C18 and C8. Moreover, phenyl columns can give a change in selectivity towards some molecules in comparison with conventional C18 columns; because the phenyl bonded stationary phase form π–π interactions, which may exploit differences in molecular shape or molecular electronic properties (Mezine, Zhang, Macku, & Lijana, 2003).

Baseline resolution of all compounds was not achieved, even when working with initial mobile phases with a high content of water and using step gradients. A better separation may be achieved by using a longer column, but would result in longer analysis time. However, the selectivity by the SRM acquisition on the

Table 1
Precursor and product ions in the APCI mass spectra and retention time (RT) for standards analysed by MS/MS.

Compound	Precursor ion (*m/z*)	Product ions		RT (min)
		Quantifier (*m/z*)	Qualifier (*m/z*)	
Vitamin D_3	385.3	259.2	159.1	7.3
Vitamin D_2	397.3	159.1	201.0	7.3
Vitamin D_3-[$^2H_3$]	388.3	259.2	162.1	7.3
Desmosterol	367.3	161.1	109.0	8.0
Ergosterol	379.3	159.1	145.1	9.0
Lathosterol	369.3	215.0	135.0	9.2
7-dehydrocholesterol	367.3	159.1	145.1	9.3
Cholesterol	369.3	161.1	147.1	10.0
Lanosterol	409.4	191.1	109.0	10.5
Cycloartenol	409.4	109.0	94.9	11.4

triple quadrupole made it possible to separate all of the analytes in 13 min giving a total run time of 28 min including cleaning and re-equilibration of the column. Furthermore, linearity studies and addition of internal standard assured the reliability of the method. Since lanosterol and cycloartenol have the same mass and are ionised giving $[M+H—H_2O]^+$, we injected these substances separately to be sure of the retention time. Similarly, we confirmed the retention times of desmosterol/7-dehydrocholesterol and lathosterol/cholesterol.

3.3. Mass spectrometry ionisation

All mass spectra were acquired by atmospheric pressure chemical ionisation (APCI) in positive mode, because it provided the highest signal intensity. ESI mode was also tested, but the sensitivity was very low for vitamin D_3 and the sterols. In fact, the sterols could not even be seen and the sensitivity of vitamin D_3 was about 25 times higher in APCI mode, when a 1 μg/mL standard mixture was used. Pre-formation of ions is very important in ESI detection mode, and it is therefore difficult to detect neutral and very apolar molecules like vitamin D and other sterols. In the APCI detection mode, solvent molecules form reagent ions first, followed by gas-phase ion–molecule reactions of reagent ions with analytes (Gao, Zhang, & Karnes, 2005). Therefore is APCI a much better ionisation technique for neutral and apolar substances like vitamin D and sterols.

MS2 scan spectra of 7-dehydrocholesterol and vitamin D_3 are shown in Fig. 2A and Fig. 2C, respectively.

The molecular ion could not be observed in APCI spectra for the sterols, e.g. missing the peak at 385.3 for 7-dehydrocholesterol (Fig. 2A). However, all sterols gave an intense ion corresponding to a loss of water from the sterol molecule, as reported in other studies (Cañabate-Díaz et al., 2007; Lu, Zhang, Wu, & Shi, 2007; Rozenberg et al., 2003). For 7-dehydrocholesterol, this results in a peak at 367.3 (Fig. 2A). Vitamin D_3 and vitamin D_2 also loses water but the molecular ion is still present in the spectrum as seen for vitamin D_3 in Fig 2C.

All compounds were analysed in selected reaction monitoring (SRM) mode aiming to improve sensitivity. In addition SRM can eliminate interfering peaks with different precursor to product ion fragmentations and thereby improving selectivity. Selectivity is always critical when analyzing plant material. In addition, the monitoring of multiple SRM pairs for a single analyte adds confidence to the identification of the analyte and provides further information for identification based on the relative intensities. The $[M—H_2O]^+$ ion was the most intense ion for all sterols and was chosen as the precursor ion. The molecular ion was chosen for the vitamins. Product ion scans were performed and two product ions were found for each precursor ion to provide both a quantifier and a qualifier ion. The product ion scans for 7-dehydrocholesterol and vitamin D_3 are shown in Fig. 2B and Fig. 2D, respectively. The most intense product ions were chosen for SRM.

3.4. MS/MS parameters

The most important parameters of the APCI interface and MS were vaporiser temperature, corona current, nebuliser and gas flow. It is generally claimed that increased vaporiser temperature leads to improved desolvation and hence better ionisation efficiency (Zarrouk, Carrasco-Pancorbo, Zarrouk, Segura-Carretero, & Fernández-Gutiérrez, 2009). However, the fact that high temperatures can decompose labile analytes has to be considered; especially vitamin D_2 was found to be sensitive to changes in vaporiser temperature, with reduced sensitivity above 325 °C. A significant decrease in sensitivity and stability of the signal was observed with corona currents above 5 μA. The gas flow had most influence on the signal intensity of the parameters studied. The capillary voltage and gas temperature had almost no impact on the signal intensity. The optimised values can be seen in section 2.6.

3.5. Selection of internal standard

An internal standard is used to eliminate analytical errors due to: 1) losses of sterols/vitamin D during extraction and separation and 2) signal variation during ionisation. Consequently, the selection of internal standard is important. It is necessary to choose a standard that closely resembles the structure of the analyte, and ideally it has to be commercially available. The internal standards most commonly used in sterol determinations are betulin, cholestane, 6-ketocholestanol, 5β-cholestan-3α-ol (epicoprostanol) and 5α-cholestan-3β-ol (dihydrocholesterol) (Lagarda et al., 2006). In this study, very low sensitivity of epicoprostanol, dihydrocholesterol and 6-ketocholestanol was observed. Therefore, these compounds are not well suited as internal standards for APCI-MS in this study. It has been noted that the response in APCI-MS is related to the sterol structure. Saturated sterols with no double bounds in the ring, like 6-ketocholestanol, epicoprostanol and dihydrocholesterol, have been shown to exhibit low response factors compared to unsaturated sterols (Ruibal-Mendieta et al., 2004).

The use of internal standard for the determination of vitamin D is essential because of the former mentioned reversible isomerisation with the corresponding previtamin D, though limited due to saponification at room temperature (Jakobsen, Clausen, Leth, & Ovesen, 2004). For determination of vitamin D_3, vitamin D_2 is the preferred internal standard and vice versa if vitamin D_2 has to be determined (CEN, 2008). This is not the best approach when vitamin D_2 and vitamin D_3 occur simultaneously as can be the case in plants. Deuterium-labeled compounds are ideal internal standards for quantification by LC–MS. Deuterated standards are not commercially available for all compounds, but a compound with a structure similar to the target analytes can be used as a surrogate. Deuterated vitamin D_3 was used in this study as an internal standard for both vitamin D_2 and vitamin D_3 and the sterols. It is absent from food and resembles vitamin D_2 and vitamin D_3 as well as the sterols. No cross-talk interference between vitamin D_3 and vitamin D_3-[$^2H_3$] was observed.

3.6. Method validation

The selectivity of the method was studied by extraction of blank samples as described in section 2.7, because plant samples without sterols were not available. Interfering compounds were not detected. LOD is an important question, when taking into account the role of sterols and vitamin D as micronutrients in foods. We found the LOD to be between 2 ng/g and 8 ng/g fresh weight for the substances tested, when a sample size of 0.4 g freeze-dried material was used. This LOD is significantly lower than in other studies using APCI-MS (Martínez-Vidal et al., 2007; Zarrouk et al., 2009). For instance Zarrouk et al. (2009) obtained a LOD for the sterols between 0.24–1.03 mg/kg corresponding to 240–1030 ng/g.

A plot of the ratio peak area of the compounds analysed/peak area of internal standard versus the ratio amount of compounds/amount of internal standard gave a linear response with regression coefficients ranging between 0.995 and 0.999. Linearity could not be determined for desmosterol and lathosterol due to impurities in the standard preparations.

The standard addition method was used to test the accuracy of the method. Three spiking levels were added to a known sample mass, and then they were carried through the entire procedure. The recoveries determined are presented in Table 2. Recoveries

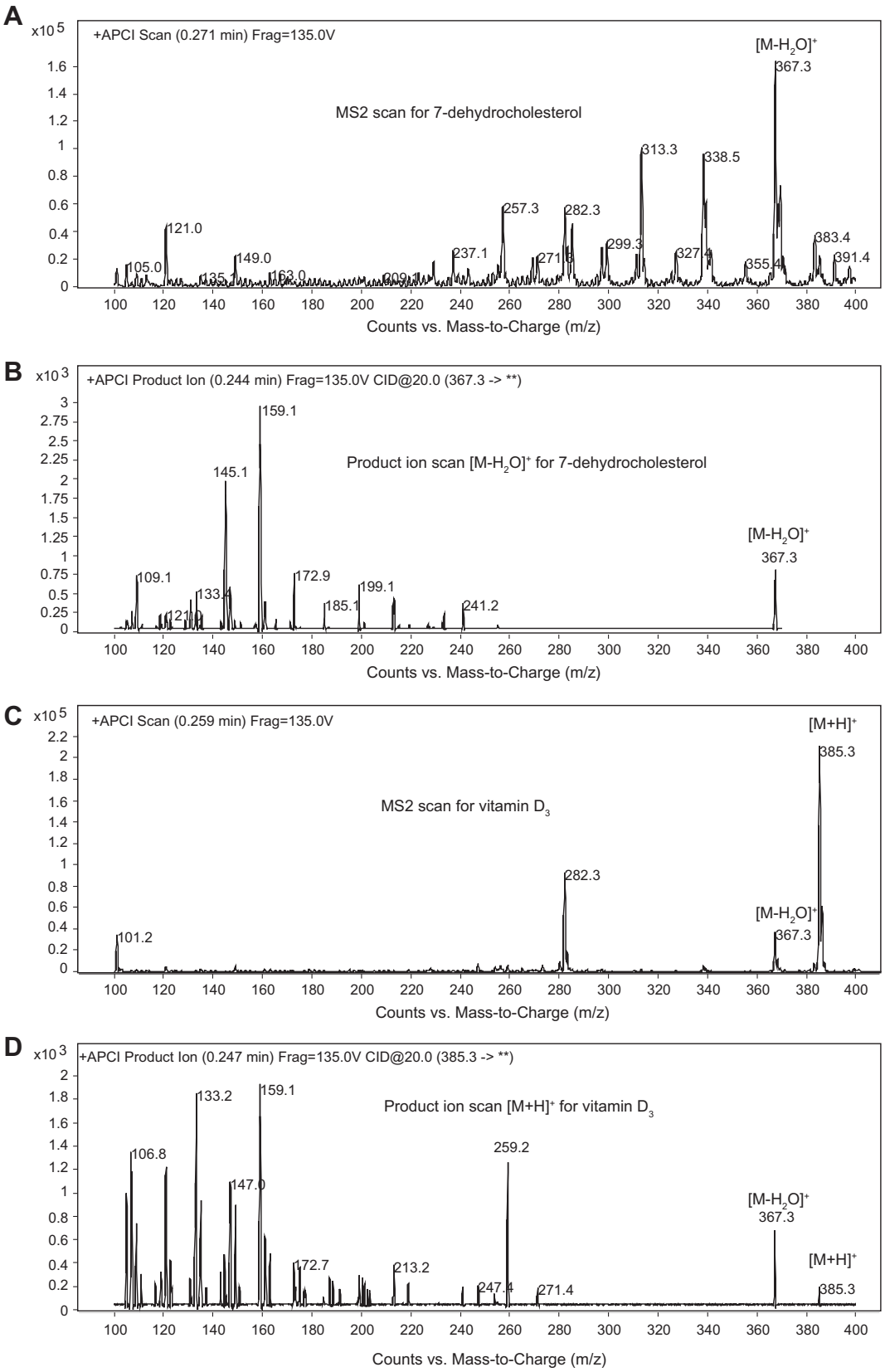

Fig. 2. Examples of APCI MS2 scans and product ions scans. (A) MS2 scan for 7-dehydrocholesterol, (B) Product ion scan for 7-dehydrocholesterol, (C) MS2 scan for vitamin D_3, (D) Product ion scan for vitamin D_3.

ranged from 101% to 114% in the inter-day repeatability test. The recoveries were in all cases above 100% and this may indicate evaporation of solvent at some stage or impurities in the standards. However, when the recoveries were corrected by the internal

Table 2
Data from recovery tests. The test is carried out by analyzing 3 analyses in 3 analytical runs carried out on different days. The inter-day reproducibility is calculated as the relative standard deviation (RSD) between the runs.

	Spiked amount (ng/g fresh weight)	Recovery %	RSD%
Vitamin D_3	37.5	103	12
	125	112	11
	500	109	6
Vitamin D_2	37.5	101	10
	125	109	9
	500	109	3
7-dehydrocholesterol	37.5	114	7
	125	108	8
	500	108	7
Ergosterol	37.5	108	12
	125	101	3
	500	105	5
Vitamin D_3-[$^2H_3$]	250	104	7

standard, recoveries under or very close to 100% were observed. The inter-day reproducibility ranged from 3% to 12%. This was acceptable and suggests that matrix-effect is not significant. The inter-day reproducibility is comparable with other studies that determines sterols in food matrices by APCI-MS (Lu et al., 2007; Mezine et al., 2003; Zarrouk et al., 2009)

3.7. Vitamin D_3 and its likely sterol precursors in plants

The method allowed to screen for vitamin D_3 and its likely precursors i.e. 7-dehydrocholesterol, cholesterol and cycloartenol in plant material. Results from before and after UV treatment of various plants are listed in Table 3, while chromatograms from before and after UV treatment for 7-dehydrocholesterol and vitamin D_3 in *S. glaucophyllum* Desf. can be seen in Fig. 3.

Interestingly, all plants studied did contain cholesterol but in various amount, the cholesterol amount was not dependent on UV treatment. As indicated in Table 3, we experienced interfering compounds eluting close to cycloartenol, which made it impossible to quantify this sterol in some of the plant extracts. This may be due to other lipophilic compounds in the extract eluting close to cycloartenol. The interferences were only observed in some plants, and not in the reference material *Spinacia oleracea* L. No interferences were observed for vitamin D_3, 7-dehydrocholesterol and cholesterol. In future work it might be necessary to purify the extracts by normal-phase semi-preparative HPLC before analysis by LC–MS/MS.

The results presented in Table 3 and Fig. 3 differ from those of Curino et al. (1998) who identified both 7-dehydrocholesterol and vitamin D_3, by electron impact MS, in *S. glaucophyllum* Desf. cultured in the dark. Though, there have been contradicting results concerning the dependence on light for vitamin D_3 formation in plants. Aburjai et al. (1996) detected 7-dehydrocholesterol by HPLC with UV detection, but not vitamin D_3 in *S. glaucophyllum* Desf. callus and cell cultures grown without UV exposure. However, vitamin D_3, but no 7-dehydrocholesterol, was identified after light exposure (Aburjai et al. 1996). The level of vitamin D_3 in the UV-treated samples found here is comparable to what has previously been found (Aburjai et al., 1996). Vitamin D_3 has earlier been identified in *S. lycopersicum* L. by ^1H NMR and MS (Aburjai et al. 1998), but to our knowledge the present report is the first time that the precursor 7-dehydrocholesterol is identified. Previous studies on vitamin D_3 in *S. lycopersicum* L. did not investigate the effect of growth conditions on vitamin D_3 formation. The present study demonstrates that UV light is capable of inducing vitamin D_3 formation in *S. lycopersicum* L.. We did not find vitamin D_3 in *C. annuum* L., *P. sativum* L. or *S. bicolor* (L.) Moench but we did find 7-dehydrocholesterol in *C. annuum* L.

P. sativum L. and *S. bicolor* L. belong to the Fabaceae and the Poaceae family, respectively. Horst et al. 1984 identified vitamin D_3 by UV absorption and mass spectrometry in *Medicago Sativa* L. (Fabaceae) at a concentration of approximately 0.63 ng/g. This is slightly lower than the detection limit in this method, and this might be the reason why we do not find vitamin D_3 in *P. sativum* L.. A lower detection limit (ng/g) can be achieved by weighing in a larger sample. In comparison Horst et al. (1984) used 800 g *M. Sativa* L. to detect 0.63 ng/g vitamin D_3. The Poaceae are monocots, in contrast with the Solanaceae and Fabaceae families, which are eudicots. The monocots are separated from all other angiosperms, and it is likely that there might be a difference between monocots and eudicots in respect to the capability of vitamin D_3 and 7-dehydrocholesterol synthesis. However, vitamin D_3-like activity has been identified in *Trisetum flavescens* (L.) P.Beauv. (Poaceae) by feeding trials with rachitic chickens (Peterlik et al., 1977).

The results for *S. glaucophyllum* Desf. and *S. lycopersicum* L. suggest that a photolytic reaction is involved in the formation of vitamin D_3 in plants, as in animals. Further experiments with a variety of plants grown under different conditions e.g. exposure to UVB light and heat needs to be carried out in order to clarify the factors that influence vitamin D_3 synthesis in plants.

4. Conclusion

The present paper reports a rapid and effective LC–APCI-MS/MS procedure for carrying out analysis of sterols and vitamin D in plant material. The method was validated with a satisfactory result. The method can be used to study the biosynthesis of vitamin D_3 in plant material. The method may easily be extended to cover other sterols and to cover other complex biological matrices, e.g. food samples. In a pilot study we identified that *S. glaucohyllum* Desf. and *S. lycopersicum* L. produced vitamin D_3 by UV-treatment. These findings, due to UV-treatment of plants, are of great interest

Table 3
Content (μg/g) of 7-dehydrocholesterol, vitamin D_3, cholesterol and cycloartenol in control and UV-treated plants.

Variety	Treatment	7-dehydrocholesterol (μg/g)	Vitamin D_3 (μg/g)	Cholesterol (μg/g)	Cycloartenol (μg/g)
Solanum glaucophyllum Desf.	Control	0.67	–	60	34
	UV	1.26	0.21	68	57
Capsicum annuum L.	Control	0.03	–	8.3	15
	UV	0.03	–	7.1	8
Pisum sativum L.	Control	–	–	1.1	26
	UV	–	–	0.8	43
Sorghum bicolor (L.) Moench	Control	–	–	5.9	ID*
	UV	–	–	6.6	ID*
Solanum lycopersicum L.	Control	0.47	–	56	ID*
	UV	0.23	0.09	45	ID*

* ID = identified

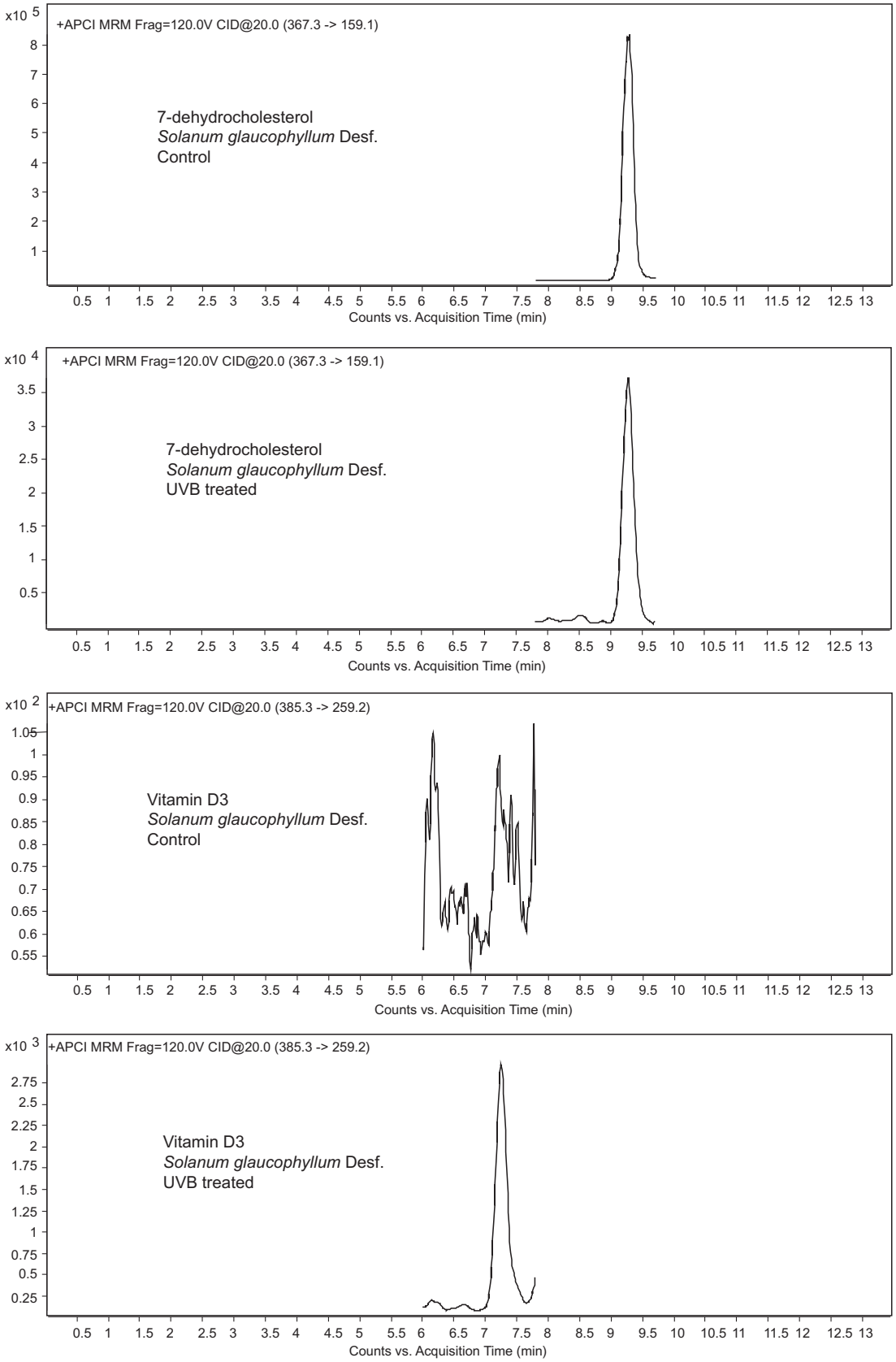

Fig. 3. Chromatograms of 7-dehydrocholesterol and vitamin D_3 in UV treated and control (nonUV treated) *Solanum glaucophyllum* Desf.

to assess the pathway for vitamin D_3 in plants, which in the future may generate plants as a vitamin D source. The method developed is a necessary step towards being able to study the biosynthesis and content of vitamin D in greater details in further research.

Acknowledgments

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Paper II

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Seasonal Variation of Provitamin D_2 and Vitamin D_2 in Perennial Ryegrass (*Lolium perenne* L.)

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ABSTRACT: Ergosterol (provitamin D_2) is converted to vitamin D_2 in grass by exposure to UV light. Six varieties of perennial ryegrass (*Lolium perenne* L.) were harvested four times during the season, and the contents of vitamin D_2 and ergosterol were analyzed by a sensitive and selective liquid chromatography tandem mass spectrometry method. Weather factors were recorded, and a principal component analysis was performed to study which factors were important for the formation of vitamin D_2. The results suggest that a combination of weather factors is involved and that the contents of ergosterol and vitamin D_2 change more than a factor of 10 during the season. These results demonstrate that grass potentially can be a significant source of vitamin D for grazing animals and animals fed on silage and hay.

KEYWORDS: Vitamin D_2, ergosterol, perennial ryegrass (*Lolium perenne* L.), liquid chromatography tandem mass spectrometry (LC-MS/MS), seasonal variation, principal component analysis

INTRODUCTION

Ergosterol (Figure 1) is a cell membrane component specific to fungi and can therefore be used as a measure of fungal growth in plant material.[1] Ergosterol is also the provitamin of vitamin D_2 (Figure 1), and small amounts of vitamin D_2 can be found in plants contaminated with fungi. The conversion to vitamin D_2 occurs by exposure of the plant material to UV light of wavelengths below 315 nm where the previtamin is formed. The previtamin D_2 undergoes spontaneous thermal rearrangement afterward to vitamin D_2. Food sources of vitamin D_2 are limited and include wild mushrooms,[2] plants,[3] milk, and butter.[4]

The main function of vitamin D in vertebrates is the maintenance and regulation of calcium homeostasis. Vitamin D is therefore critical for a healthy skeleton, and deficiency causes rickets in growing animals and osteomalacia in adult animals. Grass could be a significant source of this vitamin for grazing animals and animals fed on silage and hay, but despite the importance of grass in livestock feeding, very few studies on the vitamin D_2 content exist. One reason for this may be limitations in the analytical methods available to quantitate vitamin D_2 and ergosterol. Most publications on vitamin D_2 in grass and hay date 50−80 years back.[5−16] These studies used biological assays to determine the vitamin D activity. Biological assays are based on the ability of vitamin D to cure rickets in vitamin D-deficient rats.[17] These methods are time-consuming, imprecise, and cannot distinguish between different vitamin D compounds. Traditional chemical methods for vitamin D use high-performance liquid chromatography (HPLC) followed by UV detection with a diode array detector (DAD). These methods are in general both sensitive and selective, but analysis of vitamin D in complex matrices can be problematic.[18] Especially, analysis of grass can be challenging due to lipophilic contaminants present. The techniques to determine vitamin D have improved significantly in recent years, and problems with incomplete resolution of compounds in complex samples can be overcome by coupling chromatographic separation with mass spectrometry (MS). The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) makes it feasible to investigate vitamin D with less sample preparation, even in complex samples.[19]

The aim of this study was to identify which factors are important for the formation of vitamin D_2 in grass. We analyzed the content of ergosterol and vitamin D_2 in six varieties of *Lolium perenne* L. (perennial ryegrass) by a sensitive and selective LC-MS/MS method and investigated the seasonal variation, that is, the importance of precipitation and sun on the vitamin D_2 content.

MATERIALS AND METHODS

Plant Material and Sampling. The experiment was conducted in Bredeløkke, South-East Zealand, Denmark (55°20′N, 12°23′E), on a fine Cambisol soil (FAO soil group) containing 23% coarse sand, 39% fine sand, 17% silt, 19% clay, and 1.7% humus, pH 6.8. Six perennial ryegrass varieties (Foxtrot, Tivoli, Turandot, Telstar, Indiana, and Kimber) were sown in plots (8.0 m × 1.5 m) on June 25, 2009. The sowing density was identical to the optimal values assessed and used by DLF-Trifolium A/S, Denmark. Plots were drilled lengthwise with 10 drills per plot 120 mm apart and fertilized at seed sowing with 250 kg/ha (21:3:10:4 $N:P_2O_5:K_2O:SO_3$). During the spring and early summer 2010, the plots were fertilized using 500, 400, and 350 kg/ha of the same fertilizer and once with 160 kg/ha K_2SO_4, equaling a total N treatment of 315 kg/ha/year. Data obtained in this investigation correspond to measurements based on cuts in the first year (2010) after sowing. The plots were cut on June 4, July 15, September 2, and November 10. All cuts were carried out at 6 cm above ground level with a Haldrup plot harvester (Haldrup, Løgstør, Denmark). Samples for vitamin D_2 and ergosterol measurements were taken (around 100−200 g fresh weight)

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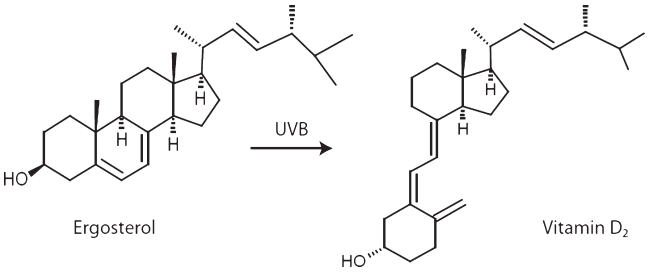

Figure 1. Conversion of provitamin D_2 (ergosterol) to vitamin D_2 by exposure of UV-B light.

and stored frozen at −20 °C until freeze drying. The grasses were finally crushed and homogenized well in a blender. The homogenized samples were stored at −20 °C under a nitrogen atmosphere until analysis.

Analysis of Vitamin D_2 and Ergosterol in Perennial Ryegrass. The analytical method and the equipment used to determine ergosterol and vitamin D_2 in perennial ryegrass have previously been described.[20] Small modifications of the sample preparation procedure were made to weigh in a larger sample size. Essentially, a larger extraction volume was used together with a larger solid phase column and a preparative HPLC step. In short, the freeze-dried plant material (2.5 g ± 0.1 g) was mixed with 15 mL of 60% potassium hydroxide in water, 30 mL of 96% ethanol, and 0.2 g of sodium ascorbate. One hundred microliters of 0.4 μg/mL vitamin D_2-[$^2H_3$] (Isosciences, King of Prussia, PA) in *n*-heptane was added to each flask. Saponification was performed overnight at room temperature (approximately 18 h) by stirring on a magnetic stirrer. The mixture was then transferred to a separation funnel with 45 mL of water and subsequently extracted with 20% ethyl acetate in *n*-heptane (v/v) (1 time with 100 mL, followed by two times of 75 mL). The combined extracts were washed with two times water to be sure that the water was free from alkali; this was confirmed with a pH strip. The extracts were evaporated to dryness in a rotary evaporator at 30 °C. The residue was redissolved in 5 mL of 1% 2- propanol in *n*-heptane (v/v) for solid phase cleanup. Clean-up was performed by solid phase extraction on a 2 g Isolute silica column (IST, Mid Glamorgan, United Kingdom) using a vacuum manifold. These columns were activated with 20 mL of *n*-heptane before the 5 mL sample extract was loaded. After they were washed twice with 10 mL of 0.5% 2-propanol in *n*-heptane (v/v), the bound compounds were eluted with 30 mL of 6% 2-propanol in *n*-heptane (v/v). The solvent was evaporated, and the residue was redissolved in 400 μL of cyclohexane/*n*-heptane (50:50) containing 0.7% 2-propanol and 2.0% methyl *tert*-butyl ether. A second cleanup was performed with a semipreparative HPLC system (Waters, Milford, MA). The system consisted of a 600 controller and pump, a 717PLUS autosampler, a 996 photodiode array detector (DAD), and a 2487 absorbance detector. Empower (Waters) was used for acquisition and processing. The HPLC system was equipped with a Luna Silica 150 mm × 4.6 mm, 3 μm column (Phenomenex, Torrance, CA), and 150 μL extract was injected. Isocratic elution with cyclohexane/*n*-heptane (50:50) containing 0.7% 2-propanol and 2.0% methyl *tert*-butyl ether as a solvent and a flow of 1.2 mL/min was used. Fractions of vitamin D, and ergosterol were collected separately in a Waters Fraction Collector. Vitamin D_2 eluted at 7.7 min, and ergosterol eluted at 10.2 min. To the ergosterol fraction was added 200 μL of 40 μg/mL cholesterol-(2,2,3,4,4,6-D6, 97−98%) (Cambridge Isotope Laboratories, Inc., Andover, MA) as the instrument standard. The fractions were evaporated by nitrogen. The vitamin D_2 fraction was redissolved in 300 μL of methanol and filtered through a 0.2 μm Vectaspin Micro, centrifugal filter prior to injection (Whatman International Ltd., Maidstone, England). The ergosterol residue was redissolved in 1.5 mL of methanol. The 1.5 mL was filtered through a 0.2 μm Ultrafree-CL filter (Millipore, Billerica, MA) and further diluted 10 times prior to injection. Sample extracts were stored at −80 °C until analysis. The analysis was done on an Agilent 1200 series HPLC connected to an Agilent 6460 series Triple Quad (Agilent Technologies, Santa Clara, CA) equipped with an atmospheric pressure chemical ionization (APCI) source. The method used was essentially as described previously.[20] Vitamin D_2 (*m*/*z* 397.3) eluted at 7.5 min, *m*/*z* 69 was used as a quantifier, and *m*/*z* 107 was used as a qualifier. Ergosterol (*m*/*z* 379.3) eluted at 9.2 min, *m*/*z* 159.1 was used as a quantifier, and *m*/*z* 145.1 was used as a qualifier. The internal standard vitamin D_2-[$^2H_3$] (*m*/*z* 400.3) eluted at 7.5 min, and *m*/*z* 69 was used as a quantifier. Cholesterol-[$^2H_6$] (*m*/*z* 375.3) was used as an instrument standard for quantitation of ergosterol and eluted at 10.2 min, and *m*/*z* 167.1 was used as a quantifier. Identification of analytes was based on the comparison of their retention times and of relative abundance of the quantifier and qualifier ions ±20%. The interday reproducibility of the method, calculated as the relative standard deviation between three different days, was previously set at 10% for vitamin D_2 on a low level and 5% for ergosterol on a high level.[20]

Quantitation. Vitamin D_2 was quantitated by using vitamin D_2-[$^2H_3$] as an internal standard. A linear regression was performed between the vitamin D_2/vitamin D_2-[$^2H_3$] area ratio and the vitamin D_2/vitamin D_2-[$^2H_3$] amount ratio. Standards of 5−500 ng/mL vitamin D_2 with 50 ng/mL vitamin D_2-[$^2H_3$] were used. Deuterated ergosterol was not available, and ergosterol was therefore quantitated by external standard with cholesterol-[$^2H_6$] as an instrument standard correcting for fluctuations in the MS signal but corrected for the recovery of vitamin D_2-[$^2H_3$] during the analytical process. Standards 250−1500 ng/mL ergosterol with 500 ng/mL cholesterol-[$^2H_6$] were used for quantitation. Standard stock solutions of vitamin D_2 and vitamin D_2-[$^2H_3$] were prepared in *n*-heptane. Concentrations of stock solutions of vitamin D_2 and vitamin D_2-[$^2H_3$] were assessed by measuring the UV absorption at 265 nm of dilutions in ethanol. The molar absorption coefficient (ε) in ethanol used for vitamin D_2 was 18843.[21] The standard stock solutions of ergosterol and cholesterol-[$^2H_6$] were prepared by dissolving the solid compounds in chloroform. The concentration of the stock solution was calculated taking into account the purity of the commercial standards. The reproducibility of the ergosterol standard was checked by analyzing the standard as a test sample, twice. The solutions were kept at −20 °C until analysis and regularly checked by measuring the UV absorption for vitamin D_2 and vitamin D_2-[$^2H_3$] and the MS/MS signal intensity for ergosterol and cholesterol-[$^2H_6$]. Working standard solutions were prepared from these solutions and diluted with methanol prior to analysis.

Statistical Analysis. A principal component analysis (PCA) was performed using The Unscrambler Software version 7.6 (Camo, Oslo, Norway). Data were mean centered (column means were subtracted from each matrix element) and divided by the standard deviation of the respective column. Standardizing ensures that the data are expressed in comparable units. Full cross-validation was used. The variables used were vitamin D_2 content, ergosterol content, cumulative precipitation (3 weeks before harvest), cumulative precipitation (5 weeks before harvest), cumulative hours of sun (3 weeks before harvest), cumulative hours of sun (5 weeks before harvest), average temperature (3 weeks before harvest), and average temperature (5 weeks before harvest).

RESULTS AND DISCUSSION

Weather. Temperature, precipitation, and hours of sun were recorded by the Danish Meteorological Institute at a weather station in Køge located 20 km from Bredeløkke. Bredeløkke and Køge are located close to the sea, and the two locations are expected to have quite similar weather. The cumulative weekly precipitation and hours of sun are displayed in Figure 2. The time of harvest is indicated in Figure 2 with bars. The weather before the first harvest was characterized by precipitation and sunshine.

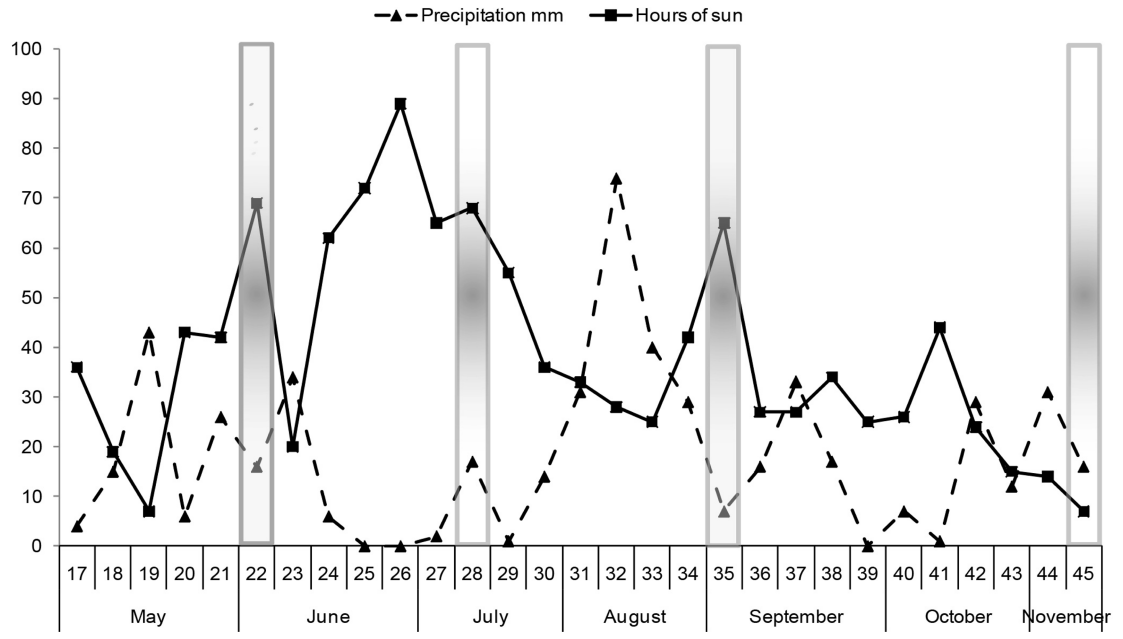

Figure 2. Precipitation (mm) and hours of sun for the period May 2010 until November 2010; the week of harvest is indicated with bars.

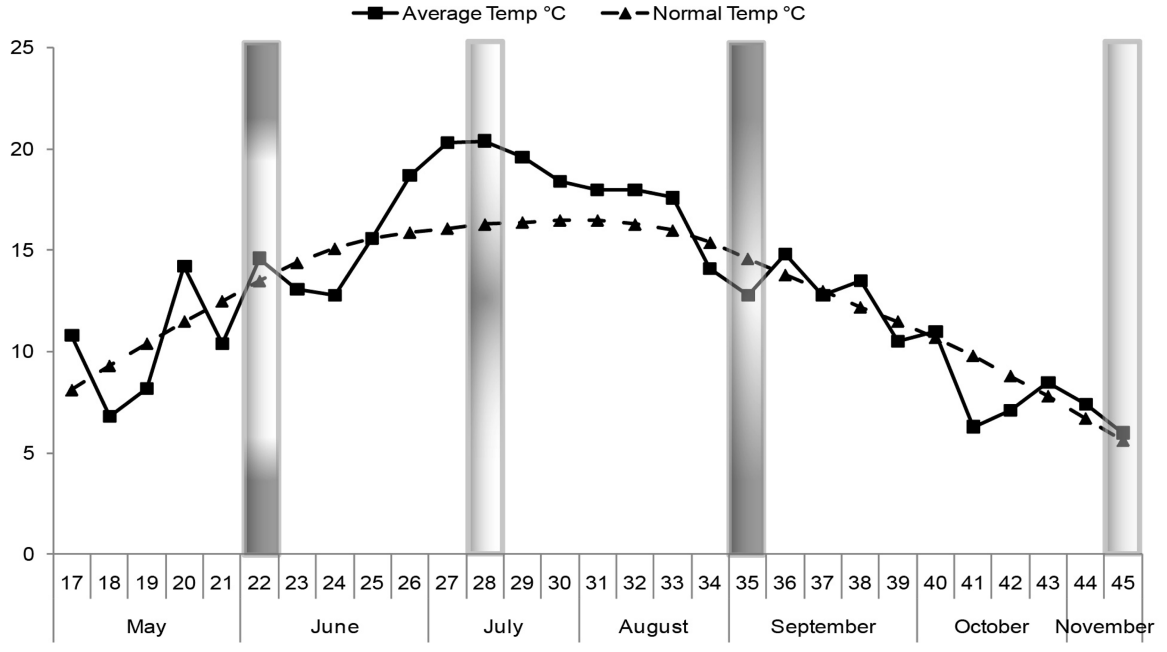

Figure 3. Normal temperature and average temperature for the period May 2010 until November 2010; the week of harvest is indicated with bars.

The period before the second harvest was characterized by a lot of sun and very little precipitation. There was heavy rainfall just before the third harvest but also periods of sun. While the weather before the fourth harvest was a mixture of sun and precipitation. The weekly average temperature in 2010 and the normal average temperature are displayed in Figure 3. The time of harvest is indicated in Figure 3 with bars. The temperature in May 2010 was fluctuating with both temperatures below and above normal. The same was the case in June 2010, with low temperatures in the beginning of the period and high temperatures in the end just before second harvest. July and early August were generally warmer than normal. September, October, and November were quite normal with temperatures reaching 5 °C in November.

Ergosterol and Vitamin D_2 in Perennial Ryegrass. The contents of ergosterol and vitamin D_2 in the six varieties of perennial ryegrass at the four harvest times are shown in Table 1. For ergosterol, the samples from September had the highest content, while those from June had the lowest. The November samples were quite high in ergosterol, whereas the July samples were in between. The content of ergosterol is similar to the content in other crops.[1,22−24] For vitamin D_2, the grass from

Table 1. Content of Vitamin D_2 (μg/kg Fresh Weight) and Ergosterol (μg/kg Fresh Weight) in Perennial Ryegrass (*L. perenne* L.)[a]

variety	harvest	vitamin D_2(μg/kg)	ergosterol (μg/kg)
Foxtrot	June	0.07	3.6×10^2
	July	1.27	2.7×10^3
	September	5.69	1.5×10^4
	November	1.08	7.2×10^3
Tivoli	June	0.07	1.8×10^2
	July	1.03	2.8×10^3
	September	6.18	1.1×10^4
	November	0.67	4.3×10^3
Turandot	June	0.19	3.4×10^2
	July	1.97	2.8×10^3
	September	3.08	4.9×10^3
	November	0.46	3.6×10^3
Telstar	June	0.14	5.6×10^2
	July	2.93	4.4×10^3
	September	3.73	7.8×10^3
	November	2.01	1.3×10^4
Indiana	June	0.11	4.2×10^2
	July	2.12	3.9×10^3
	September	2.91	7.2×10^3
	November	0.58	5.2×10^3
Kimber	June	0.41	9.5×10^2
	July	4.70	2.6×10^3
	September	6.39	1.7×10^4
	November	0.44	3.5×10^3

[a] Average of two determinations.

September had the highest content, while June samples were lowest. Generally, the July samples had a higher content of vitamin D than the November samples, despite the higher content of ergosterol in the November samples.

Perennial ryegrass is one of the most important forage crops in the temperate regions and was consequently chosen for analysis of ergosterol and vitamin D_2. It is especially valued for dairy and sheep forage systems and primarily grown for pasture and silage. Very little recent information exists on the content of vitamin D_2 in grass and in crops in general. Only two previous studies have used specific, chemical methods to determine the content of vitamin D_2 in plants.[3,24] Horst et al.[3] examined sun-cured field grown alfalfa (*Medicago sativa* L.) and found 48 μg vitamin D_2/kg, which is 7.5 times higher than the highest content found in this study. This difference may be due to loss of water by drying in the curing process. For comparison, dry matter (DM) of ryegrass hay is approximately 94.7%,[25] whereas DM of the fresh ryegrass used in this study was determined to an average of 19.2%. If we adjust for the difference in DM, the result by Horst et al.[3] is almost similar to our results. The higher vitamin D content could also be due to differences between the two varieties of crops. Magalhães et al.[24] studied the content of ergosterol and vitamin D_2 in different varieties of hop (*Humulus lupulus* L.) and found vitamin D_2 and ergosterol in only one of the varieties studied. As compared to our results, the ergosterol content at 1.84×10^3 μg/kg DM was at the same level and did not suggest that hop should be more susceptible to fungal infections than ryegrass. Our results for vitamin D_2 are significantly lower than the

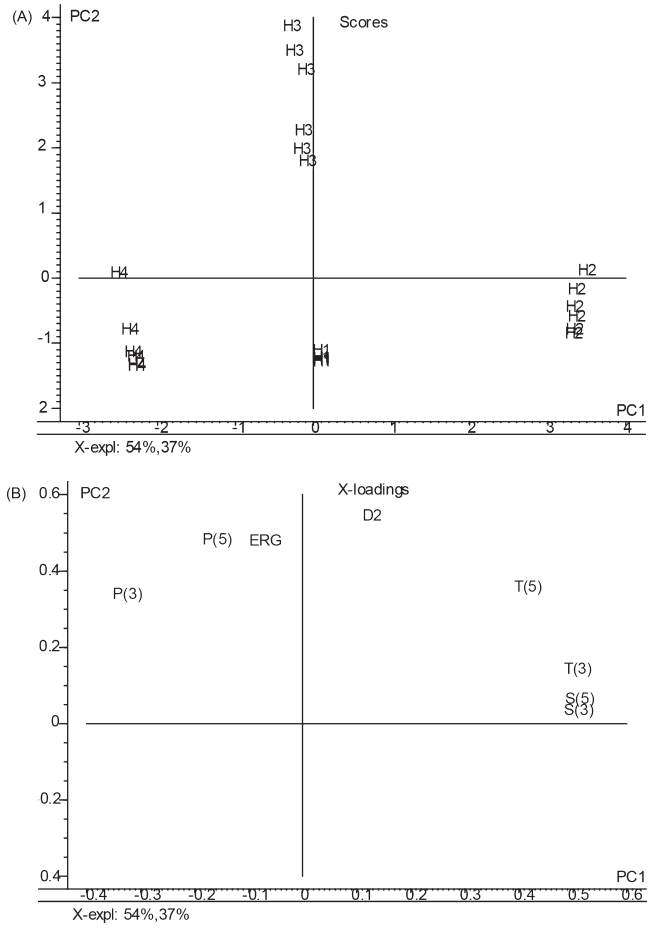

Figure 4. (A) Score plot of PC1 vs PC2, where harvest times are used as plotting symbols, H1 corresponds to the June harvest, H2 corresponds to the July harvest, H3 corresponds to the September harvest, and H4 corresponds to the November harvest. (B) Loading plot of PC1 vs PC2; variables used were vitamin D_2 content (D2), ergosterol content (ERG), cumulative precipitation 3 weeks before harvest (P3), cumulative precipitation 5 weeks before harvest (P5), cumulative hours of sun 3 weeks before harvest (S3), cumulative hours of sun 5 weeks before harvest (P5), average temperature 3 weeks before harvest (T3), and average temperature 5 weeks before harvest (T5).

1.95×10^3 μg vitamin D_2/kg DM found in hop. The hop contains ergosterol and vitamin D_2 on the same level, while vitamin D_2 in ryegrass is max 2‰ of the content of ergosterol. One explanation for a higher content of vitamin D_2 in the hop could be the difference in drying, as Portugal is located at lower latitude as compared to Denmark. Even though this only will be a part of the explanation. One of the only significant sources of vitamin D_2 is mushrooms, which synthesize vitamin D_2 by exposure to UV light in similar amounts depending on the intensity and length of irradiation.[26−28] However, the ergosterol content in mushrooms is huge in comparison to hop and ryegrass and the content of vitamin D_2 only a fraction of this.[2]

Fifty to eighty years ago, the vitamin D_2 activity of grass and hay was studied intensively by the use of biological methods.[5−16] The majority of the studies was on alfalfa (*M. sativa* L.), and most of the grasses showed activity. The vitamin D activities ranged from 0 to 3831 IU/kg, equivalent to 0−95.8 μg vitamin D/kg (1 IU of vitamin D corresponds to 0.025 μg). The average vitamin D activity found in these studies was approximately 25 μg/kg,

whereas the average content of vitamin D_2 found in the present study was 2 μg/kg. Thus, the results suggest a slight overestimation of vitamin D in previous studies using biological assays. One reason for this difference might be the analytical methods used. Our analytical LC-MS/MS method provides a high specificity and accuracy and thereby more reliable results, while the biological methods quantify the activity, that is, the ability to cure rickets. In grass, this biological activity could be due to other compounds present that increase or inhibit the activity of vitamin D. Another reason for the difference might be the difference in latitude at which the grass was grown. In this study at northern latitude while more southern latitudes will increase exposure of UV-B. In addition, most of the previous studies were done on hay, although no information of DM was given, whereas this study was done on fresh plant material.[6,8,9,11,13,14]

Seasonal Variation of Vitamin D_2. A PCA was performed to study which factors are important for the formation of ergosterol and vitamin D_2 in grass. The first principal component (PC1) explains 54% of the variance, and the second principle component (PC2) orthogonal to PC1 explains 37% of the variance. The higher components account for the remaining 9% of the variation in the data (PC3 explained 7% of the variation). Thus, the first two principal components are sufficient to describe most of the variation in the data. The score plot gives a visual image of sample variation, where we can observe how the samples are related to one another. The four harvest times are used as plotting symbols in the score plot. A clear separation between the four harvest times was observed in the score plot for PC1 vs PC2, and the score plot can be divided into four clusters representing each of the harvest times (Figure 4A). The July harvest (H2) is separated from the November harvest (H4) along PC1. The November samples (H4) is located to the left in the score plot and July samples (H2) to the right. The September harvest (H3) and the June harvest (H1) scores are almost zero in PC1 but are separated along PC2 with June samples (H1) at the lower and September samples (H3) in the upper part of the scores plot. Thus, the samples were separated by harvest time and not by variety. No trend in vitamin D_2 and ergosterol content could be observed between varieties.

The loading plot describes how the variables are related to the principal components and how much each variable contributes to each PC. The loading plot for PC1 vs PC2 (Figure 4B) shows that ergosterol content and precipitation covary. It is obvious that higher humidity of the growing season contributes to the development of mold and a higher content of ergosterol. Sun and temperature are also correlated to each other, while they are negatively correlated to precipitation and ergosterol content since these are on the opposite sides of the origin. Lower temperatures are favorable for the growth of some molds, which will enhance the content of ergosterol. This explains why temperature and ergosterol are negatively correlated. Vitamin D is located in between these two groups, indicating an influence from both sun/temperature and ergosterol/precipitation on the vitamin D content in perennial ryegrass.

Previous studies found that the vitamin D activity in general varies with the curing method and especially with exposure to sunlight.[6] However, inconsistent results were obtained regarding the importance of sun exposure, which indicates that other factors may be important,[5] as also observed in this study.

Grass as a Source for Vitamin D. Although the vitamin D_2 content reported here is quite low, it has to be taken into account that, for example, a lactating cow, as a rule of thumb, eats DM equivalent to 3.2% of their body weight each day.[29] If the weight of the cow is 700 kg, the intake will be 22.4 kg DM, which corresponds to 23 kg hay and 117 kg of the material studied here. This corresponds to an intake of 8−747 μg vitamin D_2 per day if the cows only were fed the grasses analyzed in this study. This helps explain the presence of vitamin D_2 in milk products.[4] The National Research Council (NRC) recommends that a lactating cow is provided with 30 IU/kg vitamin D per day; this is 21000 IU or 525 μg for a 700 kg cow.[30] Hence, vitamin D_2 in grass or hay can contribute to a significant amount of the needed vitamin D. The biological activity of vitamin D_2 and vitamin D_3 is generally considered equal.[30] However, Hymøller and Jensen[31] found significantly higher levels of vitamin D_3 than vitamin D_2 after ingestion of equal amounts of the two vitamins. The same difference was found in the 25-hydroxylated metabolites of the respective vitamins. This needs further investigation to evaluate grass as a source of vitamin D.

The results obtained in this study suggest that a combination of weather factors is involved in the formation of vitamin D_2 in *L. perenne* L. and that the content of ergosterol and vitamin D_2 is changing more than a factor of 10 during the season. Precipitation and high humidity are essential for ergosterol synthesis, whereas sun obviously is important for synthesis of vitamin D_2. A combination of precipitation and sun is therefore optimal for production of vitamin D_2 in perennial ryegrass. The second harvest received for instance the most sun but did not contain as much ergosterol as the third harvest and consequently not as much vitamin D_2. These findings on vitamin D_2 and ergosterol in *L. perenne* L. might be true for other varieties of grass.

Vitamin D_2 in grass is a benefit, but vitamin D_2 in grass is also linked to a risk aspect. High vitamin D_2 is associated with a high content of ergosterol, which is a measure of fungal growth. Grass is at risk of infections in the field by a number of fungi including endophytes and species such as *Fusarium* and *Claviceps*.[32] Endophytic fungi live in a symbiotic relationship with grass and can improve the resistance to stress and insects, whereas species such as *Fusarium* and *Claviceps* may give decreased yields. Fungal growth may lead to the formation of mycotoxins, which may cause diseases if consumed by animals.[32] Thus, a high content of vitamin D_2 in grass may be at the expense of the feeding quality. In general, ergosterol describes fungal biomass, which includes toxic species. Further investigation is needed to study the correlation between mycotoxins, ergosterol, and vitamin D_2.

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Paper III

Identification of vitamin D_3 metabolites and its sterol precursors in plants

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Abstract

A widespread misconception exists that only animal products contain vitamin D_3, but vitamin D_3 has been identified in several plant species, mainly belonging to the taxonomic family *Solanaceae.* However, most work has been done with non-selective methods such as bioassays. The aim of this study was to investigate changes in vitamin D_3 metabolites and its sterol precursors following UVB- and heat-treatment. A sensitive and selective LC-MS/MS method involving Diels-Alder derivatization was used to identify vitamin D_3 and its hydroxylated metabolites in *Solanum glaucophyllum* Desf.**,** *Solanum lycopersicum* L. and *Capsicum annuum* L. Vitamin D_3 and its hydroxylated metabolite were found in all UVB-treated plants. The highest vitamin D_3 content (200 ng/g dry wt.) was found in UVB-treated *Solanum glaucophyllum*, which was the only plant that also contained the dihydroxylated metabolite of vitamin D_3 in both free (32 ng/g dry wt.) and glycosylated form (17 ng/g dry wt.). Cholesterol and 7-dehydrocholesterol were found in all plants and may serve as precursors for vitamin D_3 in plants. Vitamin D_3 in plants could be of great value as a source of bio-fortification to improve the vitamin D status of the general population.

Keywords: *Solanum glacuohyllum* Desf., *Solanum lycopersicum* L., *Capsicum annuum* L.*, Solanaceae,* Vitamin D_3, Sterols, LC-MS/MS, Glycosides, Heat, UVB

1. Introduction

Vitamin D is a group of steroid hormones essential for regulating the level of calcium and phosphorus in vertebrates. Insufficient vitamin D status is a general issue for humans worldwide probably due to a lack of sun exposure (Holick, 2005). The UVB-irradiation of provitamin D_3 (7-dehydrocholesterol) in the skin of vertebrates gives a break of the B-ring to form the previtamin D_3 (**Figure 1**). The previtamin D_3 undergoes further temperature-dependent transformation to vitamin D_3 (**Figure 1**). There is a common consensus that only animal products contain vitamin D_3, despite the fact that vitamin D_3 has been identified in several plant species, mainly belonging to the taxonomic family *Solanaceae* (Aburjai et al., 1998;Aburjai et al., 1996;Boland et al., 2003;Curino et al., 1998;Horst et al., 1984;Suardi et al., 1994;Peterlik et al., 1977). The synthesis of vitamin D_3 in plants is unresolved and contradicting results concerning the dependence on UVB-light has been presented. Vitamin D_3 has in most studies been identified upon UVB-exposure (Aburjai et al., 1996;Jäpelt et al., 2011;Zucker et al., 1980;Wang et al., 2001;Björn and Wang, 2001), but vitamin D_3 synthesis without the action of UVB-exposure has also been reported (Curino et al., 1998). The hydroxylated metabolites of vitamin D_3: 25-hydroxy vitamin D_3 ($25OHD_3$) and 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) have also been claimed to exist in plants (Aburjai et al., 1998;Curino et al., 1998;Prema and Raghuramulu, 1996). In humans these are formed in the liver and kidneys respectively by two enzymatic hydroxylations, which convert vitamin D_3 into the active dihydroxylated form (**Figure 1**). In plants, a hydroxylation pathway similar to that in animals is a possibility, as enzymatic activities involved in formation of $25OHD_3$ and $1,25(OH)_2D_3$ are identified in *Solanum glaucophyllum* L. (Esparza et al., 1982). Early studies exclusively identified $1,25(OH)_2D_3$ after enzymatic hydrolysis with mixed glucosidases, which indicates that $1,25(OH)_2D_3$ mainly are found as glycosylated forms (Esparza et al., 1982;Wasserman et al., 1976;Napoli et al., 1977;Haussler et al., 1976;Hughes et al., 1977). However, this was not observed in later studies that mainly detected the free $1,25(OH)_2D_3$ (Aburjai et al., 1998;Aburjai et al., 1996;Prema and Raghuramulu, 1996). The research of vitamin D_3 in plants has so far been hindered due to limitations in the available analytical methods. Vitamin D_3 has traditionally been detected in plants by *in vivo* and *in vitro* bioassays (Wasserman, 1974;Wasserman et al., 1975;Mautalen, 1972;Walling and Kimberg, 1975) and later by HPLC with UV detection (Aburjai et al., 1998;Curino et al., 1998;Prema and Raghuramulu, 1996;Prema and Raghuramulu, 1994). These methods are currently being replaced by methods based on mass spectrometry (MS). However, direct MS analysis of

vitamin D_3 metabolites is challenging due to the small amounts present. Moreover are the ionization efficiencies of the vitamin D metabolites low in various soft ionization techniques, which together with the formation of a large number of fragments hampers sensitive and selective analysis. Attempts to increase ionization efficiency have been reported several times and Diels-Alder derivatization with reagents such as PTAD (4-Phenyl-1,2,4-triazoline-3,5-dione) has often been employed to enhance the detection response of vitamin D_3 metabolites in MS (Higashi and Shimada, 2004;Gao et al., 2005;Aronov et al., 2008;Higashi et al., 2011; Kamao et al., 2007).

The aim of this study was to obtain a better understanding of vitamin D_3 synthesis and metabolism in plants and how it changes upon UVB- and heat-treatment. Vitamin D_3 and its hydroxylated metabolites were identified in *Solanum glaucophyllum* Desf.**,** *Solanum lycopersicum* L. and *Capsicum annuum* L. by LC-ESI-MS/MS after Diels-Alder derivatization. Enzymatic hydrolysis was applied for the identification of glycoside bound forms and major plant sterols, cholesterol and 7-dehydrocholesterol were monitored by LC-APCI-MS/MS.

2. Results and Discussion

2.1 Analytical method

Plant material is a complex matrix which combined with low amounts of vitamin D_3 represents an analytical challenge. Sensitive and selective methods are therefore needed. Derivatization followed by LC-ESI-MS/MS was selected in this study due to the high sensitivity and selectivity. Derivatization is advantageous in that the molecular weight is shifted to a higher mass range, where background noise is lower. In addition the product ion spectrum of PTAD-derivatized vitamin D_3 metabolites only exhibit one major fragment, which is beneficial for sensitive selected reaction monitoring (SRM) (Ding et al., 2010). A further increase of the ionization efficiency was achieved by adding methylamine to the mobile phase (Ding et al., 2010;Higashi et al., 2008). Our goal was to quantify both sterols, vitamin D_3, 25OHD_3 and 1,25$(OH)_2D_3$ within the same extraction procedure because of the limited plant material available. Therefore a low recovery (10%) was accepted for 1,25$(OH)_2D_3$ due to poor extraction efficiency from the saponification solution. Chromatograms for 1,25$(OH)_2D_3$, 25OHD_3 and vitamin D_3 in UVB-treated *S. glaucophyllum* are shown in **Figure 2**. No interferences were present and the vitamin D_3 metabolites coeluted

with their respective deuterated standards. Two epimers, 6*S* and 6*R*, are produced by derivatization because PTAD reacts with the *s*-cis-diene system from both the α- and β-side (Ding et al., 2010). The ratio between 6*S* and 6*R* is approximately 4:1, so two peaks can be expected for each metabolite (Ding et al., 2010). Only the $25OHD_3$ epimers were fully separated and the major peak for the 6*S*-isomer was used for quantification (**Figure 2**). Both vitamin D_3 and $1,25(OH)_2D_3$ eluted as one peak, which increased sensitivity, but also broaden the peaks (**Figure 2**).

Due to the lack of glycoside standards and the diversity of the existing forms, fully determination of glycosides requires hydrolysis prior to analytical determination. Enzymatic hydrolysis was used in this study instead of conventional acid hydrolysis, to prevent acid-catalyzed isomerization of some sterols including 5,7-dienes such as 7-dehydrocholesterol (Dolle et al., 1988). Some disagreements between studies regarding the nature of vitamin D_3 and its metabolites may be attributed to the glucosidase preparations used. Kesselmeier et al. (1985) used β-glucosidase in the hydrolysis of steryl glycosides from oat leaves and seeds, but recent studies have not been successful using a similar hydrolysis method (Nyström et al., 2008;Moreau and Hicks, 2004). The enzymatic hydrolysis observed in early studies (Esparza et al., 1982;Wasserman et al., 1976;Napoli et al., 1977;Haussler et al., 1976;Hughes et al., 1977) may be due to impurities with small amounts of hydrolases rather than the β-glucosidase. Similar secondary activities are not present in modern highly purified enzyme preparations (Nyström et al., 2008;Moreau and Hicks, 2004). In future studies synthesis of conjugated standards of vitamin D_3 and its hydroxylated metabolites is needed to investigate this in more detail and to choose an optimal hydrolysis strategy.

2.2 Identification of vitamin D_3, $25OHD_3$ and $1,25(OH)_2D_3$

LC-ESI-MS/MS of the Diels-Alder derivates revealed the presence of vitamin D_3 and $25OHD_3$ in *S. glaucophyllum, S. lycopersicum* and *C. annuum*, which all belong to the taxonomic family *Solanaceae* (**Table 1**). Vitamin D_3 was present in both UVB- and non-UVB-treated *S. glaucophyllum* and *S. lycopersicum*, while only UVB-treated *C. annuum* contained detectable amounts of vitamin D_3 (**Table 1**). The levels presented in **Table 1** are the result of only one determination because of limited plant material available. However, the level of vitamin D_3 in UVB-treated *S. glaucophyllum* has previously been determined to 210 ng/g dry wt. (Jäpelt et al., 2011), which is similar to the present study. The amount of vitamin D_3 detected in *S. lycopersicum* in previous studies vary from 90 ng/g dry wt. to 1100 ng/g

fresh wt. (Aburjai et al., 1998;Jäpelt et al., 2011;Björn and Wang, 2001;Prema and Raghuramulu, 1996). All samples of *S. glaucophyllum* contained $25OHD_3$, whereas this compound was present only in UVB-treated *S. lycopersicum* and *C. annuum* (**Table 1**). The $25OHD_3$ content of *S. lycopersicum* has previously been determined to 150 ng/g fresh wt. (Aburjai et al., 1998), which is over 100-fold more than found in this study. However, Prema and Raghuramulu (1996) determined 22 ng/g dry wt., which is similar to the present study. The dihydroxylated metabolite ($1,25(OH)_2D_3$) was only identified in UVB-treated *S. glaucophyllum* (**Table 1**). It may also be present in *S. lycopersicum* and *C. annuum*, but below the detection limit (0.1 ng/g dry wt.). Contrary to the present study, Prema and Raghuramulu (1996) determined 100 ng/g dry wt. of $1,25(OH)_2D_3$ in *S. lycopersicum*. Apart from differences in growth conditions is the UV detection method used by Prema and Raghuramulu (1996) not as selective as the MS method used in the present study. Compared to the present study has a significantly higher content been reported for both vitamin D_3, $25OHD_3$ and $1,25(OH)_3D_3$ in *S. glaucophyllum* cell cultures (Aburjai et al., 1996). Formation of $1,25(OH)_2D_3$ has been shown to be greatly influenced by culture conditions and especially availability of calcium, which may explain some of the observed discrepancies (Curino et al., 2001). The difference in vitamin D_3 content observed between *in vitro* and *in vivo* conditions are in accordance with observations made with other plant secondary products (Curino et al., 2001).

2.3 Glycosylated vitamin D_3

Glycoside conjugates were found exclusively for $1,25(OH)_2D_3$ in UVB-treated *S. glaucophyllum* (**Table 1**). The glycoside content has been suggested to increase by improper drying and storage of plant material (Peterlik et al., 1977;Prema and Raghuramulu, 1994). The plant material used in this study was freeze-dried and stored at -80°C until analysis which should diminish these effects. All three hydroxyl groups in $1,25(OH)_2D_3$ are potential sites for glycosylation, but position 3 is the most likely (Grille et al., 2010). High temperatures can activate glucosyltransferase (Madina et al., 2007), but the temperature effect on glycosylations was not investigated in the present study. Glycosides usually consist of a mixture differing in saccharide moiety and the identity of the carbohydrate moiety is a subject of further studies.

2.4 Sterols

Sterols function as regulators of membrane fluidity and permeability and act as precursors of steroids including vitamin D_3. Sterols included in this study were 7-dehydrocholesterol, cholesterol, cycloartenol, sitosterol, campesterol, stigmasterol, and lanosterol. Except for lanosterol these sterols were present in all samples, though 7-dehydrocholesterol was below limit of detection in UVB-treated *C. annuum* (**Table 2**). The contents in **Table 2** are listed in the unit μg/g dry wt. as opposed to ng/g dry wt. in **Table 1.** Cholesterol accounts for 9-12% of the quantified total sterols, which is similar to other findings in *Solanaceae* (Zygadlo, 1993), which has higher content than the typically 1-2% in other families (Moreau et al., 2002). The 7-dehydrocholesterol level is similar to what has been found elsewhere (Aburjai et al., 1996; Jäpelt et al., 2011;Björn and Wang, 2001). The total sterol content was highest in samples treated with both UVB and heat (**Table 2**). UVB and heat stress the plants, which induce a variety of biochemical changes. Especially, the ability of the membranes to maintain fluidity within an optimum range will help to determine its resistance and adaption to stress (Lurie et al., 1995;Berli et al., 2010). The fluidity is a consequence of interactions among sterols and phospholipids and heat gives an increase in sterol concentrations with a concomitant increase in the sterol:phospholipid ratio (Lurie et al., 1995;Hamada, 2001). However, no correlation could be observed between a high sterol content and a high vitamin D_3 content.

2.5 Synthesis of vitamin D_3 metabolites in plants

The present study proves the presence of vitamin D_3, 25(OH)D_3 and 1,25$(OH)_2D_3$ in *S. glaucophyllum,* and vitamin D_3 and 25(OH)D_3 in *S. lycopersicum* and *C. annuum*. However, it still needs to be fully established how these compounds are formed. The content of vitamin D_3 in the UVB-treated plants was 18-64 times higher than for the non-UV-B treated plants. It is possible that in addition to an UVB-dependent pathway an alternative minor non-photolytic reaction from 7-dehydrocholesterol to vitamin D_3 takes place in plants. The inability to detect vitamin D_3 in non-UVB-treated plants in previous studies, may be due to the use of relative insensitive assays (Aburjai et al., 1996;Jäpelt et al., 2011). The isomerization of previtamin D_3 to vitamin D_3 is a temperature-dependent reaction and an effect of heat on the vitamin D_3 content could therefore be expected. However, the effect of growth temperature was minimal. The 7-dehydrocholesterol content was 3-14 times lower in UVB-treated samples, which could be related to a conversion to vitamin D_3 upon UVB-exposure. Not all 7-dehydrocholesterol was converted to vitamin D_3, which indicates that

vitamin D_3 either is transformed inside the plant e.g. to hydroxylated metabolites or that some previtamin disappear in side reactions. Prolonged UVB-exposure will in vertebrates convert previtamin D_3 to the inactive forms lumisterol and tachysterol, protecting the organism from vitamin D toxicity (Holick et al., 1981). If similar side reactions takes place in plants remains to be established. It is also likely that 7-dehydrocholesterol could enter other pathways e.g. be transformed into cholesterol. The last step in cholesterol biosynthesis in vertebrates is the reduction of the Δ-7 double bond of 7-dehydrocholesterol by the enzyme $\Delta^{5,7}$-sterol-Δ^{7}-reductase (7DHCR) to give cholesterol (Glossmann, 2010) and similar $\Delta^{5,7}$-sterol-Δ^{7}-reductase (DWARF5) exist in plants (Schaller, 2003) (**Figure 3**). Many enzymes involved in the sterol pathway do not have absolute substrate specificity (Benveniste, 1986), and it is therefore possible that DWARF5 could act on 7-dehydrocholesterol in plants to form cholesterol. The activity of DWARF5 may control the cholesterol/7-dehydrocholesterol ratio. However, it is unknown how 7-dehydrocholesterol is formed in plants in the first place. The plant sterols, such as campesterol, stigmasterol and sitosterol, are synthesized via cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants (Ohyama et al., 2009). Cholesterol and 7-dehydrocholesterol is on the other hand synthesized via lanosterol catalyzed by lanosterol synthase (LAS) in vertebrates (Ohyama et al., 2009). Although numerous labelling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor, putative LAS genes has been identified in *Arabidopsis thaliana* (Ohyama et al., 2009;Suzuki et al., 2006;Kolesnikova et al., 2006), *Panax Ginseng* (Suzuki et al., 2006) and *Lotus japonica* (Kolesnikova et al., 2006;Sawai et al., 2006). Lanosterol may act as an alternative intermediate for the synthesis of sterols, thus cholesterol and 7-dehydrocholesterol may be formed through a pathway similar to the one known from vertebrates. Only cycloartenol was detected in the current study which is not in favour of this hypothesis. It seems that sterolmethyltransferase 1 (SMT1), that catalyzes the first methylation of cycloartenol to 24-methylene cycloartenol interfere with the accumulation of cholesterol in plants (**Figure 4**). Cholesterol accumulates in plants bearing a SMT1 null mutation, which indicates that the production of high amounts of cholesterol results from a by-pass of SMT1 (Diener et al., 2000). Labelling experiments with cycloartenol and lanosterol is needed to determine the origin of cholesterol and 7-dehydrocholesterol in plants.

The ratio between vitamin D_3 and its hydroxylated metabolites provides important information about the biosynthesis and regulation of the enzymes involved. The concentration

of $25OHD_3$ in *S. glaucophyllum* is 6-9 times lower than vitamin D_3, whereas the concentration of $25OHD_3$ and $1,25(OH)_2D_3$ is equal (**Table 1**). The concentration of $25OHD_3$ in *S. lycopersicum* is 22-28 times lower than vitamin D_3, whereas it is 6-16 lower for *C. annuum* (**Table 1**). The vitamin $D_3/25OHD_3$ ratio has previously been determined to be 7 for *S. lycopersicum* (Aburjai et al., 1998). Based on these results we propose that vitamin D_3 is synthesized first and afterwards transformed into $25OHD_3$ and that conversion of $25OHD_3$ to $1,25(OH)_2D_3$ not is as tightly regulated as in vertebrates.

3. Concluding remarks

Vitamin D_3 was identified in both *S. glaucophyllum, S. lycopersicum* and *C. annuum.* All samples of *S. glaucophyllum* contained $25OHD_3$, whereas it was present only in UVB-treated *S. lycopersicum* and *C. annuum*. The dihydroxylated metabolite was identified, both free and glycoside bound, solely in UVB-treated *S. glaucophyllum*. None of the vitamin D_3 metabolites were affected by heat-treatment. Cholesterol and 7-dehydrocholesterol was found in all plants and may serve as precursors for vitamin D_3. The biosynthesis of these sterols in plants is still unknown and labelling experiments with cycloartenol and lanosterol is needed to determine the origin. Traditionally has only animal products been regarded as a source of vitamin D_3, but the present study confirms that plants can be a source of vitamin D_3. People with a low intake of animal products typically have a high intake of fruit and vegetables making plants a good new source of vitamin D. The dietary recommendations for fruit and vegetables vary, but are in most countries approx. 600 g a day. It could provide us with an additional 0.2-12 µg of vitamin D_3 per day if all 600 g was supplied by the UVB-treated leaves analyzed in the present study. In order to put it into perspective: 0.2 µg corresponds e.g. to 100 g butter (Jakobsen and Saxholt, 2009), whereas 12 µg corresponds to e.g. 50 g salmon (Chen et al., 2007). UVB-exposure of certain plants during growth could consequently be used as a source of bio-fortification to improve the vitamin D status of the general population. It seems that especially the *Solanaceous* family, which is an important source of food for humans, contain high amounts of vitamin D_3. It is of interest to determine the distribution of vitamin D_3 compounds in various species and to identify if also the fruits contain vitamin D_3 to evaluate the potential of plants as a new source of vitamin D.

4. Experimental

4.1 Plant material

Four treatments were performed on *S. glaucophyllum*, *S. lycopersicum* and *C. annuum.* Plants were grown in growth chambers with a light/dark cycle of 16/8 (light from 4:00 to 20:00), a temperature setting of 24/17°C respectively and the light set to 200 uE m^{-2} s^{-1}. Plants of 4-6 true leaves were used for treatments. The UVB-treatment was performed with a bench lamp UVP-302-15 (Ultra-Violet Products Ltd, Cambridge, UK) with 302 nm emission length equipped with 2x15W tubes. The UVB-treatment was performed in the growth chamber, where the plants were exposed to UVB-light (in addition to normal light according to the light/dark cycle) for 30 min per day for seven days (from 6:00 to 6:30). The distance from the lamp was 1 meter from the base of the pot (50 cm from the top of the plant). For the heat treatment were plants treated for seven days at 32°C to fit with the UVB-light treatment duration. The combined UVB- and heat-treatment was performed by exposing plants to UVB-light 30 min per day at 32°C for seven days. Leaves were collected at the end of the treatments and freeze-dried (Christ Beta 1-8, SciQuip Ltd, Shropshire, UK). The samples were finally crushed and homogenized well in a blender. The homogenized samples were stored at -80 ºC under a nitrogen atmosphere until analysis.

4.2 Analysis of vitamin D_3, $25OHD_3$ and $1,25(OH)_2D_3$

The freeze-dried plant leaves (0.5g±0.1g) were mixed with 10 mL 60% KOH, 20 mL 96% EtOH, 6 mL 10% NaCl, 0.5 g sodium ascorbate and 100 µL 80 ng/mL vitamin D_3-[$^2H_3$], 25-hydroxy vitamin D_3-[$^2H_3$] and 1,25-dihydroxy vitamin D_3-[$^2H_3$] (Isosciences, King of Prussia, PA, USA) in EtOH were added to each flask. Saponification was performed over-night at room temperature. The mixture was then transferred to a separation funnel with 40 mL 10% NaCl and extracted with 3 times 30 mL 20% EtOAc in *n*-heptane (v/v). The extracts were evaporated to dryness and the residue redissolved in 5 mL 1% *iso*-PrOH in *n*-heptane (v/v) for solid phase extraction on a 500 mg silica column (Isolute, IST, Mid Glamorgan, UK). The columns were activated with 5 mL *n*-heptane and washed twice with 5 mL 0.5% *iso*-PrOH in *n*-heptane (v/v). Vitamin D_3 and $25OHD_3$ were eluted with 8 mL 6% *iso*-PrOH in *n*-heptane (v/v) and 4 mL 10% *iso*-PrOH in *n*-heptane. This eluate was adjusted to 20 mL with the extraction solvent in a volumetric flask and 4 mL was withdrawn for sterol analysis. $1,25(OH)_2D_3$ was finally eluted with 8 mL 20% *iso*-PrOH in *n*-heptane. The solvent was evaporated and the residues redissolved in 400 µL *n*-heptane for further clean-up on a semi-

preparative HPLC system connected to a fraction collector (Waters, Milford, MA, USA). The HPLC system was equipped with a Luna, CN, 150x4.6mm, 3 µm column (Phenomenex, Torrance, CA). Eluent A was 1% *iso*-PrOH in *n*-heptane and eluent B was 20% *iso*-PrOH in *n*-heptane. The gradient program was as follows: 5%B for 5 min, a linear gradient to 100% B for 20 min, 100% B isocratic for 5 min and a linear gradient back to 5% B and re-equilibration for 9 min. A flow rate of 1 mL/min and UV detection at 265 nm were used. Fractions of vitamin D_3, $25OHD_3$ and $1,25(OH)_2D_3$ were collected separately and evaporated to dryness under nitrogen. 240 µL 0.5 mg/mL PTAD in anhydrous ACN was added to the residues followed by 2 hours of mixing. All extracts were filtered prior to injection (0.2 µm Ultrafree-CL, Millipore, Billerica, MA, USA). The extracts were subsequently analyzed by LC-ESI-MS/MS. Separation and detection by MS/MS was performed using an Agilent 1200 series HPLC and Agilent 6460 series Triple Quad LC/MS (Agilent Technologies, Santa Clara, CA) equipped with a Jetstream electrospray (ESI) source. The MS was operated in positive mode and nitrogen was used as a collision gas. Separation was performed on an Ascentis Express C18 2.1x100mm 2.7 µm column (Supelco, Bellefonte, PA). Eluent A was H_2O with 0.1% HCO_2H and 5 mM CH_3NH_2 and eluent B was MeOH with 0.1% HCO_2H and 5 mM CH_3NH_2. The gradient program was as follows: 70%B for 1 min, a linear gradient to 95%B for 7 min, a linear gradient to 100%B for 2 min followed by isocratic elution for 4 min and finally 5 min equilibration. The $[M+CH_3NH_3]^+$ adduct ion was used as precursor ion for all vitamin D compounds (**Table 3**). MassHunter Workstation software (version B.01.04, Agilent Technologies, Santa Clara, CA) was used for instrument control and data acquisition. The vitamin D_3 metabolites were quantified by using their deuterated form as internal standards. Limit of detection (LOD) was 20 pg/g dry wt. for vitamin D_3 and $25OHD_3$ and approx. 100 pg/g dry wt. for $1,25(OH)_2D_3$.

4.4 Analysis of sterols

The 4 mL extract withdrawn for sterol analysis was evaporated and the residue redissolved in 400 µL cyclohexane/*n*-heptane (50:50) containing 0.7% *iso*-PrOH and 2.0% MTBE for semi-preparative HPLC. The HPLC system was equipped with a Luna Silica 150x4.6 mm, 3 µm column (Phenomenex, Torrance, CA). Isocratic elution with cyclohexane/*n*-heptane (50:50) containing 0.7% *iso*-PrOH and 2.0% MTBE as a solvent and a flow of 0.8 mL/min was used. Diode array detection at 200-320 nm was used. Two fractions: 1) cycloartenol, lanosterol and 2) campesterol, cholesterol, stigmasterol, sitosterol, 7-dehydrocholesterol were collected separately. Cholesterol-(2,2,3,4,4,6-D6, 97-98%) (Cambridge Isotope Laboratories, Inc

Andover, MA, USA) was added as an instrument standard. Fraction 1 was taken up in 5 mL methanol and fraction 2 in 300 µL, which was further diluted 10 times. The sterols were analyzed as described in Jäpelt et al. (2011). In addition to the sterols described there, were major plants sterols included: sitosterol, campesterol and stigmasterol. Sitosterol (*m/z* 397.3) eluted at 12.1 minutes, *m/z* 161 was used quantifier and *m/z* 81.1 was used as qualifier. Campesterol (*m/z* 383.3) eluted at 11.0 minutes, *m/z* 161 was used as quantifier and *m/z* 95 was used as qualifier. Stigmasterol (*m/z* 395.3) eluted at 11.3 minutes, *m/z* 161 was used as quantifier and *m/z* 83 was used as qualifier.

4.5 Analysis of glycosides

The freeze-dried plant material (0.5g±0.1g) was mixed with 30 mL EtOAc and 0.5 g sodium ascorbate and shaken over-night at room temperature in a vibrating tube shaker. The tubes were subsequently centrifuged at 2000 *g* for 5 min and the organic layer withdrawn. This was repeated twice, but with 30 min of shaking. The combined extracts were evaporated to dryness and the residue was redissolved in 2.5 mL 1% *iso*-PrOH in *n*-heptane (v/v) for SPE on a 500 mg silica column (Isolute, IST, Mid Glamorgan, UK). The columns were activated with 5 mL *n*-heptane and washed twice with 5 mL 0.5% *iso*-PrOH in *n*-heptane (v/v) and 8 mL 6% *iso*-PrOH in *n*-heptane (v/v). Glycosides were finally eluted with 8 mL Me_2CO. Correct separation was confirmed with standards of vitamin D_3, $25OHD_3$, $1,25(OH)_2D_3$ and steryl glycosides (Matreya LLC, Pleasant Gap, PA). Steryl glycosides were used as a surrogate as no commercial vitamin D_3 conjugates exist. 100 µL 80 ng/mL vitamin D_3-[$^2H_3$], 25-hydroxy vitamin D_3-[$^2H_3$], 1,25-dihydroxy vitamin D_3-[$^2H_3$] (Isosciences, King of Prussia, PA, USA) in EtOH were added and the eluate was evaporated to dryness under nitrogen. The method for enzymatic hydrolysis of glycosides was adapted from (Nyström et al., 2008). In short the residue was dissolved in 50 µL DMSO before the enzyme was added in 500 µL 0.1 M citrate buffer (pH 5.0) with 5 mM sodium taurocholate. The enzyme used was Cellobiase from *Aspergillus Niger* (Sigma-Aldrich, Steinheim, Germany). This mixture was hydrolyzed over-night in a shaking water bath set at 45°C. The free vitamin D_3 metabolites were then transferred to another tube with 5 mL water and extracted with pentane:EtOAc (80:20) for 30 min. This was repeated two times. The combined extracts were evaporated and dissolved in 400 µL 1% *iso*-PrOH in *n*-heptane for semi-preparative HPLC. The liberated vitamin D_3 metabolites were analyzed similar to described in 4.3.

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Figures and Tables

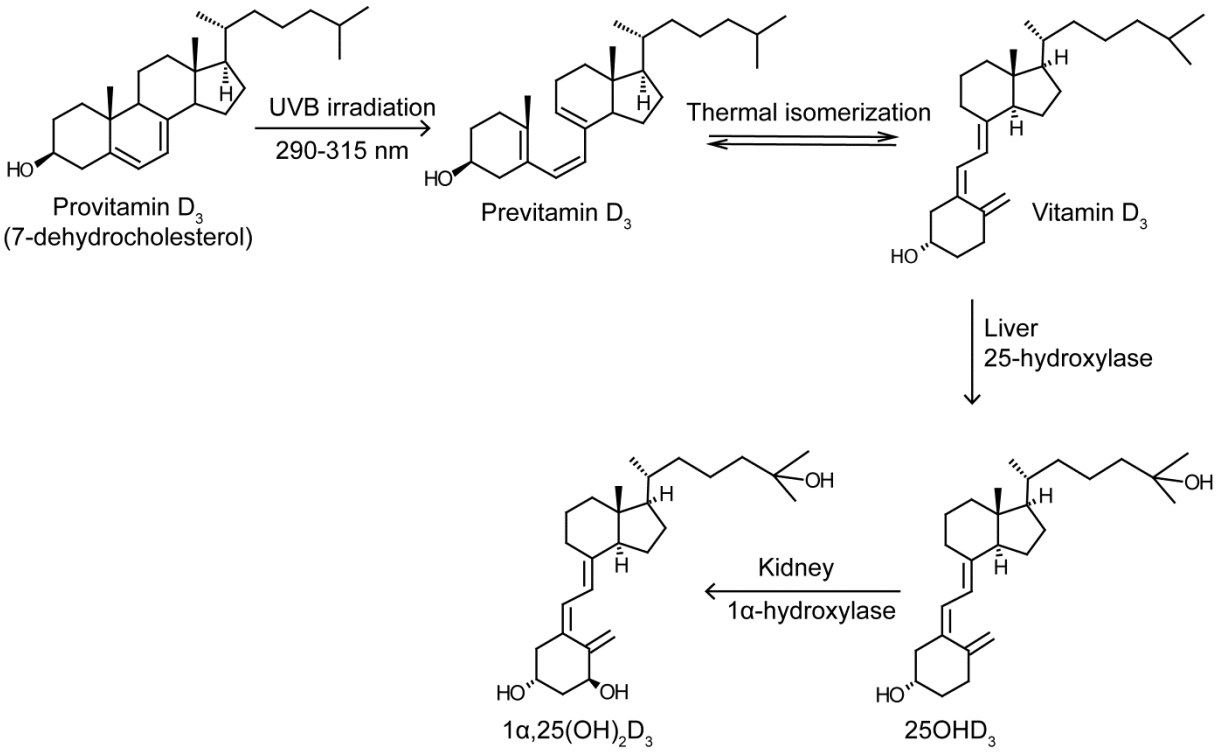

Figure 1.Synthesis and activation of vitamin D. Vitamin D_3 is synthesized in the skin upon UVB-irradiation. UVB-irradiation of provitamin D_3 (7-dehydrocholesterol) in the skin gives a break in the B-ring to form previtamin D_3, which undergoes thermally induced rearrangement to vitamin D_3. Vitamin D_3 is transported to the liver where it is hydroxylated at C-25 by the enzyme 25-hydroxylase producing $25OHD_3$, which is the major circulating form in vertebrates. The $25OHD_3$ is hydroxylated a second time at C-1 in the kidneys to the active metabolite $1\alpha,25(OH)_2D_3$.

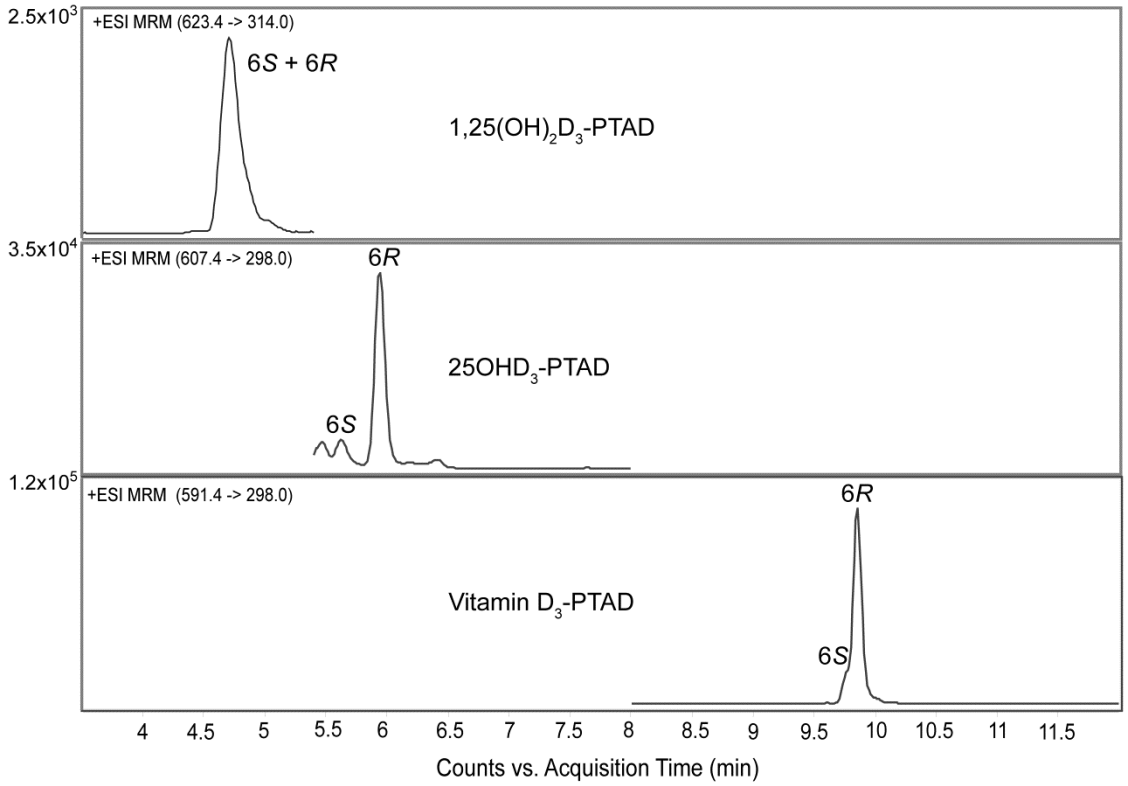

Figure 2. Chromatograms for Diels-Alder derivatized vitamin D_3 and its hydroxylated metabolites in UVB-treated *Solanum glaucophyllum* Desf. PTAD = 4-phenyl-1,2,4-triazoline-3,5-dione.

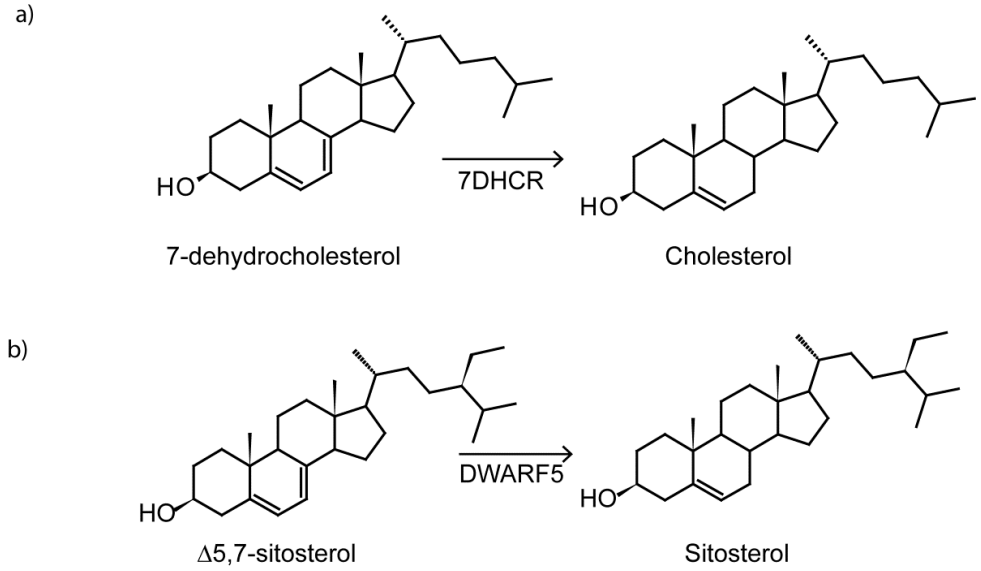

Figure 3. a) The conversion of 7-dehydrocholesterol to cholesterol by the action of Δ5,7-sterol-Δ7-reductase (7DHCR) b) The conversion of Δ5,7-sitosterol into sitosterol by the action of DWARF5.

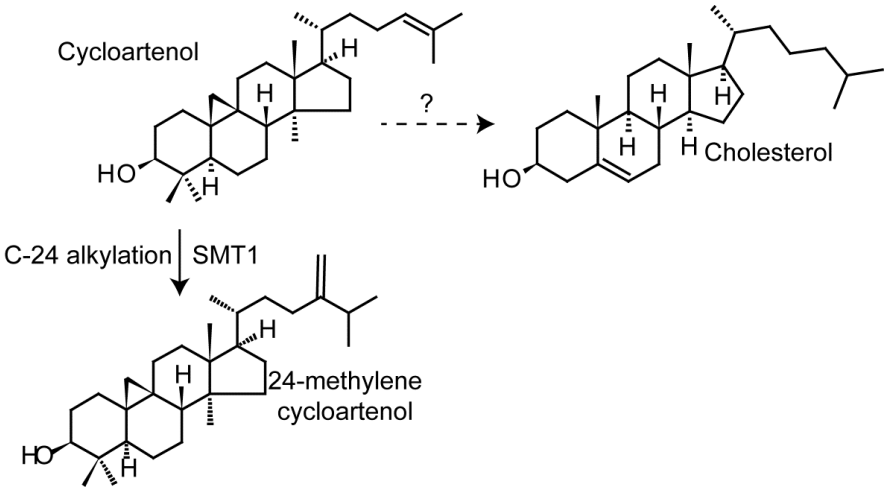

Figure 4. Action of sterol methyltransferase 1 (SMT1) on cycloartenol to yield 24-methylene cycloartenol. It is unknown how cholesterol is formed, but cholesterol has been shown to accumulate in plants bearing a SMT1 null mutation.

Table 1. Vitamin D_3, $25OHD_3$ and $1,25(OH)_2D_3$ in *Solanum glacucohyllum* Desf., *Solanum lycopersicum* L., and *Capsicum annuum* L. (ng/g dry wt. leaves). The plants were grown at elevated temperature (Heat), UVB treated during growth (UV) or a combination (Heat + UV)

	Treatment	Vitamin D_3	$25OHD_3$	$1,25(OH)_2D_3$	Glycosylated $1,25(OH)_2D_3$
Solanum glaucophyllum Desf.	UV	200	31	32	17
	Heat	5.5	0.8	<0.1	n.a.
	Heat + UV	100	11	12	n.a.
	Control	3.2	0.8	<0.1	<0.1*
Solanum lycopersicum L.	UV	100	4.3	<0.1	<0.1*
	Heat	2.3	<0.02	<0.1	n.a.
	Heat + UV	110	3.8	<0.1	n.a.
	Control	1.7	<0.02	<0.1	<0.1*
Capsicum annuum L.	UV	2.9	0.5	<0.1	<0.1*
	Heat	<0.02	<0.02	<0.1	n.a.
	Heat + UV	6.3	0.4	<0.1	n.a.
	Control	<0.02	<0.02	<0.1	<0.1*

n.a. = not analyzed, n = 1, * = LOD estimated to be the same as for $1,25(OH)_2D_3$

Table 2. 7-dehydrocholesterol, cholesterol, cycloartenol, sitosterol, campesterol and stigmasterol in *Solanum glaucophyllum* Desf., *Solanum lycopersicum* L., and *Capsicum annuum* L. (µg/g dry wt. leaves). The plants were grown at elevated temperature (Heat), UVB treated during growth (UV) or a combination (Heat + UV)

	Treatment	*S.glaucophyllum*	*S.lycopersicum*	*C. annuum*
7-dehydrocholesterol	UV	1.6	0.09	-
	Heat	16	0.59	0.33
	Heat + UV	2.7	0.17	0.09
	Control	23	0.40	0.17
Cholesterol	UV	30	1.3	3.0
	Heat	67	6.9	3.4
	Heat + UV	82	18	2.9
	Control	67	10	1.8
Cycloartenol	UV	82	91	54
	Heat	185	41	48
	Heat + UV	135	242	81
	Control	144	29	24
Sitosterol	UV	173	7.2	5.2
	Heat	220	23	5.1
	Heat + UV	347	12	3.2
	Control	265	6.3	3.6
Campesterol	UV	27	5.7	2.1
	Heat	45	5.6	2.3
	Heat + UV	146	1.0	1.4
	Control	42	4.5	2.1
Stigmasterol	UV	29	28	2.3
	Heat	32	22	3.8
	Heat + UV	50	40	2.2
	Control	32	18	2.5
Total sterols*	UV	343	133	67
	Heat	565	99	63
	Heat + UV	763	313	91
	Control	573	68	34

*Total sterols refer only to the cumulative amount of the sterols studied here, n = 1

Table 3. Selected reaction monitoring segments, precursor and product ion transitions, collision energies and retention time for vitamin D_3 metabolites

Segment	Compound	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Retention time (min)
1	$1,25(OH)_2D_3$	623.3	314	15	4.8
1	$1,25(OH)_2D_3$-[$^2H_3$]	626.3	314	15	4.8
2	$25OHD_3$	607.3	298	15	6.0
2	$25OHD_3$-[$^2H_3$]	610.3	298	15	6.0
3	D_3	609.3	298	15	9.9
3	D_3-[$^2H_3$]	612.3	298	15	9.9

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Paper IV

Review: Vitamin D in plants – biosynthesis, occurrence and function

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Abstract

Could plants be a vitamin D source for humans? The answer to this question requires knowledge on how vitamin D synthesis takes place in plants and how it is regulated. Several plant species are known to cause calcium intoxification in grazing animals, which has been attributed to the presence of vitamin D_3-like activity. Despite the evidence that vitamin D_3 exist in plants are plant products generally not considered a source of vitamin D_3. Research into the presence and regulation of vitamin D_3 in plants is extremely limited. This review summarizes the current knowledge on sterol biosynthesis leading to provitamin D_3. The quality of the detection methods used to study vitamin D and related compounds are discussed in parallel with the content of vitamin D and its hydroxylated metabolites in higher plants and in plankton. The work on vitamin D_2 in plants will also be reviewed. The possible biological functions of vitamin D_3 in plants are considered. Additionally, it is discussed whether vitamin D_3 in plants is bound as conjugates e.g., esters, glycosides and acetylated glycosides.

Keywords: Plants, synthesis, content, vitamin D, 25-hydroxy vitamin D, 1,25-dihydroxy vitamin D, sterols, detection, function

1. Introduction

Vitamin D is a group of fat-soluble vitamins, which is mainly represented by cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) (**Figure 1**). They differ structurally in the C-17 sidechain, where vitamin D_2 has a double bound and an additional methyl group. Exposure to UV-light below 315 nm of the provitamin D_2 (ergosterol) in fungi and provitamin D_3 (7-dehydrocholesterol) in the skin of vertebrates gives a break in the B-ring to form the previtamin D (**Figure 2**). The previtamin D undergoes thermally induced transformation afterwards to vitamin D. Vitamin D requires activation and is first hydroxylated in the liver at carbon 25 by the enzyme vitamin D 25-hydroylase in a non-restricted reaction to yield 25-hydroxyvitamin D (25OHD) (**Figure 3**). In passing through the kidneys 25OHD is hydroxylated at the α-position of carbon 1 by 1α-hydroxylase to generate the active metabolite 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) (**Figure 3**). Vitamin D is essential for normal skeleton development and maintenance of calcium homeostasis. The known nutritional deficiency diseases of vitamin D are rickets in children and osteomalacia in the elderly. Vitamin D insufficiency has been linked to increased risk of cancer (Lappe et al., 2007;Holick, 2004), diabetes (Hyppönen et al., 2001;Pittas et al., 2007), autoimmune diseases (Cantorna and Mahon, 2004) and cardio-vascular diseases (Kendrick et al., 2009). There is, therefore, a growing awareness about vitamin D as a requirement for optimal health. The insufficiency is probably due to a lack of sun exposure, which is the main source of vitamin D for humans during summer.

Vitamin D_3 is generally considered absent from plants, but this may not be true. Grazing animals in several parts of the world develop calcinosis from consuming specific toxic plants. The hypothesis is that excess vitamin D_3 stimulates calcium absorption producing hypercalcemia and deposition of calcium in soft tissue including aorta, heart, kidney, intestine and uterus (Mello, 2003). The symptoms are loss of weight, bent forelegs, reduced milk production, increased heart rate and possibly death (Rambeck et al., 1979). If plants contain vitamin D_3, they also have the potential to serve as a source of this important vitamin. However, research into the synthesis, presence and regulation of vitamin D_3 in plants is extremely limited. This is partially due to the dogma that plants do not synthesize vitamin D and partially because of limitations in the analytical methods. Analytical technologies have improved much recent years and it is now possible to investigate vitamin D in plants in much more details.

This review will serve as starting point for those not familiar with vitamin D in plants. It summarizes the current knowledge on sterol biosynthesis leading to 7-dehydrocholesterol and vitamin D_3. The quality of detection methods used to study vitamin D and related compounds will be discussed in parallel with the findings of vitamin D_3 and its hydroxylated metabolites in higher plants and in plankton. A discussion of the occurrence of vitamin D_3 as conjugates in plants and the impact on bioavailability and biological activity is included. Besides vitamin D_3, small amounts of vitamin D_2 occur in plants (Horst et al., 1984). Vitamin D_2 may be formed from fungal ergosterol, when exposed to sun during growth and in the curing process and work in this area will also be summarized. Finally, the possible biological function of vitamin D in plants will be considered.

2. Sterols – precursors of vitamin D

Sterols act as precursors of steroids, including vitamin D hence ergosterol is a provitamin for vitamin D_2 and 7-dehydrocholesterol for vitamin D_3. Sterols are made up of a tetracyclic system with a long side chain at C-17 (**Figure 4**). The four rings are designated A, B, C and D as shown (**Figure 4**). The sterols can in general be divided in C27, C28 and C29 sterols, where the C28 have a methyl group at C-24 and the C29 an ethyl group at C-24. C27 sterols are typical in vertebrates, whereas the C28 and C29 are typical in plants. Sterols are components of membranes and have a function in regulation of membrane fluidity and permeability (Piironen et al., 2000). Cholesterol in animals and ergosterol in fungi fulfil this role, whereas the plant kingdom in comparison produces very diverse sterols. More than 200 sterols have been found in plants, but sitosterol, campesterol and stigmasterol normally predominates (Lagarda et al., 2006). To understand how vitamin D_3 synthesis takes place we need to understand how its sterol precursors are formed.

2.1 Sterol biosynthesis - presqualene

The sterols are built through a complex biosynthesis from the common C5 isoprene units isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The intermediate to form these isoprene units is mevalonic acid (MVA) (Rodrıguez-Concepción and Boronat, 2002). MVA itself is a product of acetate metabolism. Three molecules of acetyl-CoA are initially combined to make the branched-chain ester (*S*)-3-hydroxy-3-methylglutaryl-CoA (*S*-HMG-CoA). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the two step reduction of *S*-HMG-CoA into MVA (Benveniste,

2002). MVA is then phosphorylated to MVA-5-diphosphate in two steps, which is converted to IPP by mevalonate diphosphate decarboxylase. IPP is then isomerized to its isomer DMAPP. One molecule DMAPP and two molecules IPP are then assembled into farnesyl diphosphate (C15). Finally, two molecules farnesyl diphosphate are combined to make squalene (C30). The process from MVA to squalene is the same for all eukaryotes; however large differences exist downstream from squalene (Benveniste, 2002). Cyclization of squalene is via the intermediate 2,3-oxidosqualene (**Figure 5**), which forms either lanosterol or cycloartenol via a series of enzymatic cyclizations.

2.2 Sterol biosynthesis – fungi and vertebrates

Ergosterol and cholesterol are synthesized via lanosterol catalyzed by lanosterol synthase (LAS), in fungi and vertebrates, respectively (Ohyama et al., 2009). A simplified pathway downstream from lanosterol is displayed in **Figure 6**. Cholesterol and ergosterol share the pathway until zymosterol (Lees et al., 1995, **Figure 6**). The conversion of lanosterol to cholesterol involves removal of three methyl groups, reduction of double bonds and migration of double bond in lanosterol to a new position in cholesterol (Waterham et al., 2001). The biosynthesis from lanosterol to cholesterol is a 19-step process that requires nine different enzymes (Risley, 2002) and the sequence of reactions in the cholesterol biosynthetic pathway may vary (Waterham et al., 2001). Alternate routes exist because reduction of the C-24,25 double bond on the hydrocarbon side chain of the sterol ring by sterol Δ^{24}-reductase can occur at multiple points in the pathway, giving rise to various intermediates (Bae and Paik, 1997). These intermediates, with or without a double bond in the hydrocarbon side chain, can serve as substrates for other enzymes in the pathway.

2.3 Sterol biosynthesis - plants

The plant sterols, such as campesterol, stigmasterol and sitosterol, are synthesized via cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants (Ohyama et al., 2009). A simplified biosynthetic pathway downstream from cycloartenol is shown in **Figure 7**. Campesterol are 24-methyl analogues of cholesterol, whereas sitosterol and stigmasterol are 24-ethyl analogues. Besides the 24-methyl and 24-ethyl sterols is cholesterol, as a surprise to many, also present in plants at low levels. Cholesterol often accounts for 1-2% of plant sterols, but can account for as much as 5% in selected plant families (Moreau et al., 2002). Especially, *Solanaceae* species include high levels of cholesterol (Moreau et al.,

2002;Zygadlo, 1993;Whitaker, 1991;Whitaker, 1988;Jäpelt et al., 2011b). The last step in cholesterol biosynthesis in vertebrates is the reduction of the Δ-7 double bond of 7-dehydrocholesterol by the enzyme Δ-7 sterol reductase to give cholesterol (Glossmann, 2010). A similar Δ-7 sterol reductase (DWARF5) exists in plants (Schaller, 2003). Many enzymes involved in the biosynthesis of sterols do not have absolute substrate specificity (Benveniste, 1986) and it is, therefore, possible that DWARF5 could act on 7-dehydrocholesterol in plants to form cholesterol. The activity of DWARF5 may control the cholesterol/7-dehydrocholesterol ratio. However, it is not clear how 7-dehydrocholesterol and cholesterol is formed in plants in the first place. Although numerous labelling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor, were putative LAS genes recently identified in *Arabidopsis thaliana* (Ohyama et al., 2009;Suzuki et al., 2006;Kolesnikova et al., 2006), *Panax Ginseng* (Suzuki et al., 2006) and *Lotus japonica* (Kolesnikova et al., 2006;Sawai et al., 2006). This suggests that LAS genes might be widespread among eudicots, which include 70% of angiosperm species. Lanosterol may act as an alternative intermediate for sterol synthesis thus cholesterol and 7-dehydrocholesterol could be formed by a pathway similar to the one known from vertebrates (**Figure 6**). The proportion of cholesterol and the major plant sterols sitosterol and campesterol has been shown to be controlled by the activity of sterol methyltransferases (SMTs). SMTs catalyze the transfer of two carbon atoms from *S*-adenosyl methionine to make the 24-alkylations and are considered important regulatory steps in the biosynthesis of sterols in plants (Schaller, 2003). There are two classes of SMTs: SMT1 and SMT2 (**Figure 7**). SMT1 catalyzes the first methylation of cycloartenol to 24-methylene cycloartenol, and seems to interfere with the accumulation of cholesterol in plants. Overexpression of SMT1 in tobacco reduced the content of cholesterol (Sitbon and Jonsson, 2001;Holmberg et al., 2002), but no change in the cholesterol level has also been observed (Schaeffer et al., 2000). In *Arabidopsis thaliana* plants, bearing a SMT1 knockout, cholesterol was the major sterol, composing 26% of total sterols, compared with 6% in wild-type plants (Diener et al., 2000). The accumulation of cholesterol in plants bearing a SMT1 knockout indicates that the production of high amounts of cholesterol results from a by-pass of SMT1. It remains to be discovered whether 7-dehydrocholesterol and cholesterol in plants is formed from lanosterol and/or cycloartenol.

2.4 Analysis of sterols

Sterol analysis is essential to investigate the biosynthesis of vitamin D_3 in plants. Analysis of sterols is usually based on solvent extraction followed by hydrolysis to liberate esters (Piironen et al., 2000). Further clean-up of the extracts is usually needed to remove interfering compounds (Lagarda et al., 2006). This can be achieved by thin layer chromatography (TLC), column chromatography or more recently by solid phase extraction (SPE). Fractionation by TLC and column chromatography is time-consuming and not suitable for routine analysis and can usually be replaced by SPE providing faster fractionation using less solvent (Lagarda et al., 2006). Sterols are typically measured by capillary gas chromatography (GC) as trimethylsilyl (TMS) ether derivates (Piironen et al., 2000) detected by flame ionization detection (FID) (Brufau et al., 2006;Liu et al., 2007;Phillips et al., 2005) or mass spectrometry (MS) (Nyström et al., 2007;Toivo et al., 2001). MS is crucial to identify sterols and for evaluating peak purities. The main disadvantage with GC is that it requires time-consuming derivatization (Razzazi-Fazeli et al., 2000;Lu et al., 2007). High performance liquid chromatography (HPLC) offers compared to GC the advantage of analysis without derivatization and gentler conditions suitable for thermally unstable sterols. GC is generally considered superior over HPLC for sterol analysis (Lagarda et al., 2006), but progress in HPLC technology may change these roles. In HPLC the detection can be carried out by UV (200-210 nm) (Careri et al., 2001;Sanchez-Machado et al., 2004), evaporative light scattering (Hong et al., 2007) and MS (Jäpelt et al., 2011b;Lu et al., 2007;Mezine et al., 2003; Ruibal-Mendieta et al., 2004;Rozenberg et al., 2003;Cañabate-Díaz et al., 2007). Most sterols only adsorb UV in the 200-210 nm range. Low sensitivity is, therefore, observed by UV detection due to low transparency of most organic solvents. UV detection is also non-specific for sterols and a complete separation is therefore needed, which is difficult even though columns with particle sizes of 1-2 μm have been introduced which may improve resolution of co-eluting sterols (Lu et al., 2007). Problems with incomplete resolution can be resolved by hyphenating chromatographic separation to selective detection by MS. However, the analysis of sterols by conventional electrospray ionization (ESI) is difficult due to the dynamics of the electrospray process. Atmospheric pressure chemical ionization (APCI) has generally been found to be more effective (Jäpelt et al., 2011b;Lu et al., 2007;Martínez-Vidal et al., 2007). A significant challenge is that the occurrence of the various sterols span several orders of magnitude where the major sterols such as sitosterol and campesterol is between 10-200 μg/g, whereas minor sterols are present at less than 0.01 μg/g (Schrick et al., 2011). Consequently, the analytical methods require a huge dynamic range.

3. Biological methods for identification of vitamin D_3 and its metabolites in plants

3.1 Biological assays for vitamin D and its hydroxylated metabolites

Vitamin D has traditionally been detected in plants by *in vivo* and *in vitro* bioassays (Boland et al., 2003). The official method for vitamin D was for many years the line test using animals (Parrish and Richter, 1979). Either a rat or a chicken was put on a vitamin D deficient diet until the animal developed rickets. After the animals developed rickets, they were fed plant material or extracts and it was estimated how much the plant was able to cure rickets by staining with silvernitrate to show deposition of calcium salts or by radiographic pictures. The line test is time-consuming as it takes about five weeks and it runs with low precision. Results from the biological assays are given in international units (IU), the conversion ratio is 1 IU to 0.025 µg vitamin D. Increased calcium absorption and elevation in blood calcium has also been used as an indicator for the existence of vitamin D-related compounds in plants (Basudde and Humphreys, 1976;Mautalen, 1972;Walling and Kimberg, 1975). The accuracy of these methods may be discussed and the fact is that the amount of quantified vitamin D corresponds to the total vitamin D activity independent of the specific metabolites and their possible difference in activity. In chickens with a high strontium intake is the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ blocked by suppressing 1α-hydroxylase activity (Wasserman, 1974). The inhibitory effect of strontium can be overcome by the administration of $1,25(OH)_2D_3$, but not by $25OHD_3$ and vitamin D_3. This could be used as a more specific methods to show if a plant contains a $1,25(OH)_2D_3$-like compound. Studies of calcium absorption in nephrectomized rats, with as suppressed 1α-hydroxylase activity, have also been used to detect vitamin D activity (Walling and Kimberg, 1975). These methods potentially give a false positive result, due to compounds interfering with vitamin D metabolism or calcium absorption or to other compounds present e.g., calcium and phosphorus that increase or inhibit the activity of vitamin D.

3.2 Identification of vitamin D and its metabolites by biological assays

Most work has been made on *Solanum glaucophyllum* Desf. that causes calcinosis in cattle in Argentina (Mello, 2003). Controlled studies with various animals including rabbits (Mautalen, 1972;Dallorso et al., 2008;Humphreys, 1973), chickens (Wasserman et al., 1976a; Weissenberg et al., 1989) and rats (Basudde and Humphreys, 1976;Uribe et al., 1974) verified that *S. glaucophyllum* or an extract caused an increased absorption of calcium and

phosphorus. Improvement of bone mineral density in a rat osteoporosis model (von Rosenberg et al., 2007) and promotion of bone resorption *in vitro* has also been demonstrated (Puche and Locatto, 1974). *S. glaucophyllum* reverse the inhibitory effect of dietary strontium (Wasserman, 1974;Weissenberg et al., 1989) and increase calcium absorption after administration in nephrectomized rats (Walling and Kimberg, 1975). This demonstrates that *S. glaucophyllum* can affect calcium absorption without the participation of 1α-hydroxylase, which indicates that the calcinogenic factor is $1,25(OH)_2D_3$ rather than vitamin D_3 itself. *Cestrum diurnum* L. and *Trisetum flavescens* Beauv. cause calcinotic diseases very similar to *S. glaucophyllum* (Wasserman et al., 1975).

4. Identification of vitamin D_3 in plants

4.1 Chemical analysis of vitamin D_3 in plant material

Analysis of vitamin D in plant material is not an easy task. Plants are in general a difficult matrix because of interferences, which combined with very low contents of vitamin D makes the analysis particular challenging. Extraction followed by proper sample preparation is, therefore, crucial for reliable analysis. Saponification followed by liquid-liquid extraction is typically used where cold saponification is preferred over hot saponification due to the reversible and a temperature-dependent equilibration between vitamin D and pre-vitamin D (Buisman et al., 1968;Hanewald et al., 1968). The extraction of free vitamin D present in the non-saponifiable matter is usually performed by liquid/liquid extraction using non-polar organic solvents (CEN, 2008). Precaution has to be taken to prevent decomposition due to exposure to light and oxidation during sample handing and extraction (CEN, 2008). Purification of the extracts is typically needed either by SPE or semi-preparative HPLC to avoid contamination of the analytical column by other co-extracted substances e.g., chlorophyll and other lipophilic pigments (Jäpelt et al., 2011b). In previous studies on vitamin D in plants liquid-liquid extraction and column chromatography or/and HPLC were used for purification (Rambeck et al., 1979;Morris and Levack, 1982;Esparza et al., 1982;Prema and Raghuramulu, 1994;Curino et al., 1998;Skliar et al., 2000;Curino et al., 2001). GC was the first chromatography principle used to replace the biological assay. However, due to the poor volatility vitamin D needs to be derivatized to trimethylsilyl (TMS) ethers (Bell and Christie, 1973). Vitamin D undergoes thermal cyclization in a GC split/splitless injector (>125°C) resulting in formation of the corresponding pyro and isopyro compounds (Yeung and Vouros, 1995). Vitamin D gives rise to double peaks as a result of this thermic rearrangement (Yeung

and Vouros, 1995). As single ion monitoring (SIM) sensitivity for vitamin D is decreased by isomerization and GC-MS methods require a derivatization step they did not gain use for routine assays. However, early studies on vitamin D_3 in plants did use GC for identification (Rambeck et al., 1979;Suardi et al., 1994). HPLC with UV detection at 265 nm is used in official methods for vitamin D in food (CEN, 2008;Staffas and Nyman, 2003) and has been used in recent studies on vitamin D in plants (Prema and Raghuramulu, 1994;Curino et al., 1998;Curino et al., 2001;Prema and Raghuramulu, 1996;Aburjai et al., 1996;Aburjai et al., 1997;Aburjai et al., 1998). Nevertheless, these methods are laborious as they need a high degree of purification and are not totally specific. Analysis of vitamin D in complex matrices such as plants can be especially challenging and occasionally co-eluting interferences occur (Byrdwell, 2009). Today the method of choice is LC-MS exploiting the high selectivity and sensitivity to unravel the complexity of plants extracts. MS have been used for identification of vitamin D_3 several previous studies, but not coupled to LC (Curino et al., 1998;Skliar et al., 2000;Aburjai et al., 1996). However, one problem with MS detection is relatively low sensitivity. The ionization efficiency of vitamin D is low in the most commonly used electrospray ionization (ESI) methods because of few polar functional groups (Dimartino, 2007). Atmospheric pressure chemical ionization (APCI) is today the most widely used ionization technique for vitamin D analysis (Jäpelt et al., 2011b; Byrdwell, 2009;Dimartino, 2007) as it is a much more efficient ionization technique for neutral and apolar substances such as vitamin D. Atmospheric pressure photoionization (APPI) is also suitable for detection of vitamin D (Soldin et al., 2009). LC-MS/MS improves both selectivity and sensitivity compared to LC-MS in particular by using selected reaction monitoring (SRM). SRM increase selectivity, however, more than one transition is needed for reliable confirmation of a specific analyte, which preferable is combined with other evidence such as relative intensities of product ions in the mass spectra, accurate mass, retention time and peak shape positively to identify the compound as vitamin D (Jäpelt et al., 2011b). An internal standard is essential for quantification of vitamin D due to reversible isomerization with the corresponding previtamin D (Schlatmann et al., 1964). Internal standards are also needed to eliminate analytical errors due to losses of vitamin D during extraction and separation and signal variation in ionization in the MS (Dimartino, 2007). Deuterium-labeled compounds are ideal internal standards for quantification by MS, because of the complete resemblance with the analyte. Nuclear magnetic resonance (NMR) would offer valuable information in addition to UV and MS. NMR is a powerful tool for structure elucidation and can discriminate between compounds that only differ in terms of local chemical environment e.g., compounds with same mass, but

different locations of functional groups. However, in general NMR analyses lack sensitivity of the mass spectrometer and more purified samples are often needed (Eisenreich and Bacher, 2007). Nevertheless, ^{1}H NMR has been used for identification of vitamin D_3 in plants, but extraction of as much as 2 kg fresh plant leaves was required (Aburjai et al., 1998).

4.2 Identification of 7-dehydrocholesterol and vitamin D_3 by chemical analysis

The plant species and contents of vitamin D_3 and/or its provitamin 7-dehydrocholesterol found are listed in **Table 1** belonging to *Solanaceae* (*Solanum lycopersicum* L.*, Solanum tuberosum* L.*, Solanum glaucophyllum* Desf.*, Nicotiana glauca* Graham*, Cestrum diurnum* L. *and Capsicum annuum* L.)*, Cucurbitaceae* (*Cucurbita pepo* L.), *Fabaceae* (*Medicago sativa* L.) and *Poaceae* (*Trisetum flavescens* Beauv.). Boland et al. (2003) states that it is unexpected that independent traits can take place several times in the evolution of different families and hypothesized that the ability to synthesize vitamin D_3 is characteristic of angiosperms in general. However, this remains to be established. Although several species have been shown to contain vitamin D_3, most of them belong to the taxonomic family *Solanaceae* (Esparza et al., 1982;Prema and Raghuramulu, 1994;Curino et al., 1998;Skliar et al., 2000; Prema and Raghuramulu, 1996;Aburjai et al., 1998). The synthesis of vitamin D_3 in plants is unresolved and contradicting results concerning the dependence on light have been presented. Vitamin D_3 has in most studies been identified after UVB exposure (Jäpelt et al., 2011b; Aburjai et al., 1996;Zucker et al., 1980;Björn and Wang, 2001), but vitamin D_3 synthesis without the action of UVB has also been reported (Curino et al., 1998). The non-photolytic production of vitamin D_3 has been proposed to be an enzyme-catalyzed conversion of 7-dehydrocholesterol to vitamin D_3 via retro-ene rearrangement, enzymatic epoxidation of 7-dehydrocholesterol or through a pathway involving radical intermediates (Norman and Norman, 1993). It is possible that besides the UVB-independent pathway a more important photolytic reaction from 7-dehydrocholesterol to vitamin D_3 takes place in plants.

4. Identification of hydroxylated metabolites of vitamin D in plants

Hydroxylated metabolites of vitamin D_3 have been found in certain plants (**Table 2**) and a hydroxylation pathway similar to that in animals may be present. Enzymatic activities involved in formation of $25OHD_3$ and $1,25(OH)_2D_3$ have been identified in *S. glaucophyllum* (Esparza et al., 1982). Vitamin D 25-hydroxylase activity has been localized in the microsomes, whereas the 1α-hydroxylase activity has been localized in mitochondria and

microsomes (Esparza et al., 1982). Attempts were later made to demonstrate hydroxylation of vitamin D_3 in *Populus tremula* using ^{14}C-labelled vitamin D_3, but without success (Pythoud and Buchala, 1989). This suggests that vitamin D_3 metabolism is different from vitamin D_3-rich plants such as *S. glaucophyllum* (Pythoud and Buchala, 1989).

Analysis of the hydroxylated metabolites of vitamin D_3 represents a challenge because they exist in even lower concentrations than vitamin D_3 (**Table 2**). They have been detected in plants using both protein-binding assays (Skliar et al., 2000;Curino et al., 2001) and chemical methods (Prema and Raghuramulu, 1994;Prema and Raghuramulu, 1996;Aburjai et al., 1996; Aburjai et al., 1998). The most common chemical detection principle used has been HPLC with UV detection. Protein-binding assays, including RIA (radioimmunoassay) and RRA (radioreceptor binding assay), is widely used for analysis of 25OHD and 1,25$(OH)_2$D in clinical laboratories due to the simplicity (Hollis and Horst, 2007). RRA has been applied for identification of 1,25$(OH)_2D_3$ in *S. glaucophyllum* (Curino et al., 2001) and *Nicotiana glauca* (Skliar et al., 2000). The plant material was extracted and then purified by column chromatography. The vitamin D receptor (VDR) was harvested from chicken duodena mucosa (Skliar et al., 2000). RRA was performed with ^{3}H-1,25$(OH)_2D_3$ which compete for binding to VDR. The RRA is highly sensitive, but besides the obvious disadvantage of isolating VDR from animals, the possibility of non-specific competition or inactivation of VDR by other lipophilic compounds has to be taken into account. RIA for both 1,25$(OH)_2$D and 25OHD exist, these assays are commercialized and are widely used especially for serum samples (Hollis and Horst, 2007). RIA has been used without any prior purification for extracts and cell cultures of *S. glaucophyllum* and *C. diurnum* (Weissenberg et al., 1988). The lipophilic nature of vitamin D makes it difficult to analyze in any protein-binding assay due to solubility problems (Hollis and Horst, 2007). Matrix effects are also common usually due to lipids not found in the standard tube, but in the assay tube that compete with binding to the protein and increase the risk for false-positive results (Hollis and Horst, 2007). However, RIA can differentiate among lower and higher yielding plants in a screening process. This was demonstrated by Gil et al. (2007) who used RIA for screening of vitamin D activity in *S. glaucophyllum*. Protein-binding assays are currently being replaced by MS methods due to their specificity. However, direct LC-MS/MS analysis of especially 1,25$(OH)_2$D is challenging because of poor ionization efficiency, low concentration and an extensive product ion spectra by most soft ionization techniques. Attempts to increase ionization efficiency of the hydroxylated metabolites of vitamin D_3 have been reported several times mostly for

analysis of serum samples. Kissmeyer and Sonne (2001) developed a LC-MS/MS method that quantified the ammonium adduct of $1,25(OH)_2D$ with a LOQ of 20 pg/mL using 1 mL of serum (Kissmeyer and Sonne, 2001). Another LC-MS/MS method quantifying the lithium adduct of $1,25(OH)_2D$ with an LOD of 15 pg/mL using 0.2 mL of serum has also been developed (Casetta et al., 2010), but involved a complicated LC system. Derivatization with Cookson-type reagents has often been employed to enhance the detection response in MS (Higashi and Shimada, 2004;Gao et al., 2005;Aronov et al., 2008;Higashi et al., 2011;Kamao et al., 2007). Aronov et al. (2008) used 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), a Cookson-type reagent, to derivatize $1,25(OH)_2D$ before ESI-LC-MS/MS analysis and a LOD of 25 pg/mL using 0.5 mL of serum was achieved. Microflow LC-MS together with derivatization has also been used, which improved sensitivity 15-fold compared to normal LC when using a 0.2 mL serum sample (Duan et al., 2010). However, microflow LC has a small loading capacity that counteracts the sensitivity gain, especially when analyzing complex matrices (Duan et al., 2010).

5. Conjugates of sterols and vitamin D in plants

Sterols are found with a free 3-hydroxyl in most plant species, but sterol acetylation and sterol glycosylation happens in varying degree. The sterols are acetylated with fatty acids or conjugated with sugars, which are often also acetylated (**Figure 8**). These are all together called conjugates. Steryl esters are present in all plants, are most often localized in the cytoplasm of plant cells (Benveniste, 2002) and do typically represent a storage form of sterols (Piironen et al., 2000). Steryl glycosides usually consist of a mixture differing in saccharide moiety and especially plants from the *Solanaceae* family demonstrate an unique abundance of glycosides (Moreau et al., 2002;Potocka and Zimowski, 2008). Since glycosylation is a general metabolic process that occurs in plants are vitamin D_3 and its metabolites expected to be found as glycosides.

5.1 Analysis of conjugates

Saponification liberates esters and acetylated glycosides in free form, but fails to hydrolyze the bond between vitamin D and the carbohydrate moiety (Toivo et al., 2001). Several methods have been described for analysis of steryl glycosides in various plant matrices and both direct and indirect analysis (with or without hydrolysis) has been used (Van Hoed et al., 2008). Direct steryl glycoside analysis can be performed with GC-FID (Phillips et al.,

2005;Lacoste et al., 2009), HPLC-UV (Kesselmeier et al., 1985), HPLC-ELS (Moreau et al., 2008), GC-MS (Gutiérrez and José, 2001) and LC-MS (Rozenberg et al., 2003). However, there is a lack of conjugated standards and a large diversity of the existing forms, which makes direct analysis complicated. Acid hydrolysis has traditionally been used to release glycosidic sterols (Liu et al., 2007;Nyström et al., 2007;Toivo et al., 2001). Acid hydrolysis is typical performed under relatively harsh conditions e.g., 60 minutes at 80°C with 6 M ethanolic hydrochloric acid solution (Nyström et al., 2007;Toivo et al., 2001;Kamal-Eldin et al., 1998). Acid hydrolysis has the disadvantage that isomerization of some sterols occurs (Kamal-Eldin et al., 1998) including 5,7-dienes as 7-dehydrocholesterol (Dolle et al., 1988) and vitamin D_3 (Jin et al., 2004). Enzymatic hydrolysis with β-glucosidase is a gentler method of hydrolysis. Kesselmeier et al. (1985) used β-glucosidase in the hydrolysis of steryl glycosides in oat leaves and seeds, but recent studies have not been successful in similar hydrolysis (Nyström et al., 2008;Moreau and Hicks, 2004). The hydrolysis demonstrated by Kesselmeier et al. (1985) might be caused by activity of unknown minor hydrolases in the crude enzyme rather than β-glucosidases. Similar secondary activities are not present with the modern highly purified enzyme preparations (Nyström et al., 2008;Moreau and Hicks, 2004).

5.2 Occurrence of vitamin D conjugates in plants

Early work identified $1,25(OH)_2D_3$ in *S. glaucophyllum* after enzymatic hydrolysis with mixed glycosidases (Esparza et al., 1982;Wasserman et al., 1976b;Napoli et al., 1977;Haussler et al., 1976;Hughes et al., 1977) and in a similar study in *C. diurnum* (Hughes et al., 1977). Vitamin D_3 and $25OHD_3$ have also been identified in *S. glaucophyllum* after incubation with a glycosidase (Esparza et al., 1982). The existence of glycosides is debated and other studies mainly detected the free forms (Prema and Raghuramulu, 1996; Aburjai et al., 1996;Aburjai et al., 1998). The glycoside content might be dependent on the collection, dying and storage of the plant material (Prema and Raghuramulu, 1994;Peterlik et al., 1977). The number of glycoside units and the identity seems to differ since the glycosides from *S. glaucophyllum* and *T. flavescens* are soluble in water (Morris and Levack, 1982;Wasserman et al., 1976b;Napoli et al., 1977), while the glycoside of *C. diurnum* is less polar and soluble in a mixture of chloroform and methanol (Wasserman et al., 1976a;Hughes et al., 1977). The site of glycosylation, the type of glycosidic bond involved and the identity of the carbohydrate moiety are not completely determined. Vidal et al. (1985) isolated the $1,25(OH)_2D_3$ glycoside from *S. glaucophyllum* and found that $1,25(OH)_2D_3$ was bound to a series of fructoglucosides.

The structure was investigated through periodic acid oxidation, which demonstrated that the fructose was linked to a disaccharide unit repeating one, two or four times (Vidal et al., 1985). Esters of vitamin D_3 and the hydroxylated metabolites have not been identified in plant material.

The formation of glycosides may cause dramatic changes in the chemical, nutritional and metabolic properties of vitamin D_3 and its metabolites. Rambeck et al. (1984) studied the biological activity of mono-glycosides of $1\alpha(OH)D_3$ and vitamin D_3 and the corresponding parent molecules in bioassays using rats, chickens and quails. In rats and chickens, vitamin D_3 and the vitamin D_3 β-D-glucoside exhibited nearly equivalent activity (Rambeck et al., 1984). In contrast, the β-D-glucoside of $1\alpha(OH)D_3$ exhibited only 10% activity relative to $1\alpha(OH)_2D_3$ in all bioassays, whereas the disaccharide derivate exhibited no vitamin D activity in the chicken bioassay (Rambeck et al., 1984). No such study have been performed on $25OHD_3$ and $1,25(OH)_2D_3$. Aqueous extracts of *S. glaucophyllum* leaves preincubated with bovine ruminal fluid (De Boland et al., 1978) and ovine ruminal fluid (Esparza et al., 1983) exhibited more vitamin D activity than extracts not incubated. Later the presence of vitamin D_3 and its metabolites were identified in *S. glaucophyllum* extracts incubated with ovine ruminal fluid (Skliar et al., 1992). This indicates that vitamin D_3 and its metabolites may be liberated from its glycosides at least in ruminants. In general, improvement and development of analytical procedures are needed to study vitamin D glycosides in more details.

6. Phytoplankton and algae as an origin of vitamin D_3 in fish

Fish are known to be rich sources of vitamin D_3, but the origin of vitamin D_3 in fish has not been clarified. Photochemical production of vitamin D_3 in fish skin is doubted due to non-availability of enough UVB-light in their natural habitats combined with low amounts of 7-dehydrocholesterol in their skin (Bills, 1927;Takeuchi et al., 1991;Rao and Raghuramulu, 1997;Sunita Rao and Raghuramulu, 1996a). A non-photochemical pathway for vitamin D_3 synthesis in fish is also questionable (Takeuchi et al., 1991;Sugisaki et al., 1974;Sunita Rao and Raghuramulu, 1996b). Thus, vitamin D_3 must be of dietary origin where plankton at the base of the food chain is of prime interest as a source for animals higher in the food chain. Hence the high content of vitamin D_3 in fish may be because of accumulation in the food chain originating from plankton (Takeuchi et al., 1991;Sunita Rao and Raghuramulu, 1996a). Data for vitamin D_2 and vitamin D_3 in phytoplankton is very limited (**Table 3**). Drummond and Gunther suggested as early as 1934 that plankton could be the origin of vitamin D_3 in fish.

However, they found no antirachitic activity of phytoplankton and very low activity of zooplankton (Drummond and Gunther, 1934). Takeuchi et al. (1991) found significant amounts of both vitamin D_2 and vitamin D_3 and their provitamins in phytoplankton (**Table 3**). Since plankton usually lives at the surface of the water, vitamin D is probably synthesized by solar radiation of provitamins D (Takeuchi et al., 1991). Takeuchi et al. (1991) observed that phytoplankton caught in August were higher in vitamin D than in October and December (**Table 3**), this supports that vitamin D is synthesized from solar radiation on provitamin D. Sunita Rao and Raghuramulu (1996a) reported high concentrations of ergosterol, 7-dehydrocholesterol, vitamin D_2 and vitamin D_3 in freshwater phytoplankton (**Table 3**).

To synthesize vitamin D_3 by UVB exposure, phytoplankton should be able to synthesize 7-dehydrocholesterol if using the same pathway as vertebrates. Phytoplankton is likely to be one of the most important sources of sterols, including cholesterol, in the marine environment and the sterols found in phytoplankton display a great diversity as may be expected from the large number of algae classes and species combined with a long evolutionary history (Volkman, 2003). Red algae (*Rhodophyta*) primarily contain cholesterol, although several species contain large amounts of desmosterol. Fucosterol is the dominant sterol of brown algae (*Phaeophyta*) (Patterson, 1971). Generalizations about the sterols in most other phytoplankton e.g., diatoms (*Bacillariophyta*) and green algae (*Chlorophy*ta) cannot be made as they are much more varied. The most common sterol in diatoms are 24-methylcholesta-5,24(28)-dien-3β-ol, but cholesterol and sitosterol are also very common (Rampen et al., 2010). The green algae are very variable, they contain significantly amounts of C29 sterols (Volkman, 2003), but also cholesterol and ergosterol (Patterson, 1974). Since cholesterol and 7-dehydrocholesterol biosynthesis are closely linked, the presence of cholesterol may indicate capability of 7-dehydrocholesterol synthesis. However, this needs to be verified.

7. Vitamin D_2 in plant material

UVB-exposure of a variety of vegetables and crops in the beginning of the twentieth century yielded antirachitic activity (Hess and Weinstock, 1924), which was stated to be vitamin D_2 produced from ergosterol when exposed to UVB. Ergosterol is the predominant sterol found in fungi, but ergosterol is also the provitamin of vitamin D_2 and small amounts can be found in plants contaminated with fungi. The conversion to vitamin D_2 occurs by sun-exposure of the plant material during growth and in the curing process. Vitamin D in grass and hay was studied intensively 50-80 years back using biological assays (Thomas and Moore,

1951;Thomas, 1952;Wallis, 1939;Wallis, 1938;Wallis et al., 1958;Russell, 1929;Newlander and Riddell, 1952;Newlander, 1948;Henry et al., 1958;Steenbock et al., 1925;Moore et al., 1948;Keener, 1954). However, very little recent information exists on the content of vitamin D_2 in plants. Magalhães et al. (2007) studied the content of ergosterol and vitamin D_2 in different varieties of hop (*Humulus lupulus* L.) and found vitamin D_2 and ergosterol in only one variety. Horst et al. (1984) examined sun-cured field grown alfalfa (*Medicago sativa* L.) and found 48 µg vitamin D_2/kg. Recently, Jäpelt et al. (2011a) studied the seasonal variation of vitamin D_2 in *Lolium perenne* L. and found that the content varies more than a factor of ten during the season.

8. Biological function of vitamin D and related substances in plants

The biological function of vitamin D_3 and its metabolites in plants remains unclear. The theory is that vitamin D or vitamin D-like substances act as plant growth substances mediated by changes in calcium fluxes. Vitamin D_2 and vitamin D_3 enhance the number of adventitious roots in *Populus tremula* L.*, Populus nigras* L. (Buchala and Schmid, 1979), *Phaseolus vulgaris* L. (Buchala and Schmid, 1979;Talmon et al., 1989) and in *Phaseolus aureus* Roxb. (Jarvis and Booth, 1981). Of the metabolites of vitamin D_3, only $1,25(OH)_2D_3$ promotes adventitious rooting, but to a smaller extent than vitamin D_3 (Pythoud et al., 1986). Glycosides of several vitamin D_3 metabolites promoted rooting to the same extent as the parent metabolite (Pythoud et al., 1986). The effect of vitamin D_3 on root growth is proposed to be connected to calcium uptake and calmodulin synthesis (Talmon et al., 1989;Vega et al., 1985). Vitamin D has also been demonstrated to stimulate cell division in root meristems (Talmon et al., 1989) and calmodulin synthesis and Ca^{2+}-uptake in *P. vulgaris* root segments *in vitro* (Vega et al., 1985;Vega and Boland, 1986). Habib and Donnelly (2005) found that vitamin D_3 applied to potato plantlets stimulated Ca^{2+} uptake, but also UV exposure stimulated Ca^{2+} uptake, which may be a result of photo-dependent vitamin D_3 synthesis. It has been proposed that vitamin D_3 stimulates DNA synthesis in the tissue of root meristem at least partially through changes in intracellular calcium and calmodulin (Boland et al., 2003;Vega and Boland, 1986;Vega et al., 1989). Milanesi and Boland (2006) identified a protein similar to the vertebrate vitamin D receptor (VDR) in *S. glaucophyllum*, whether the VDR-like proteins play a functional role remains to be established.

Besides the role as plant growth substances, it has been proposed that the 7-dehydrocholesterol/vitamin D_3 system act as a sensor for UV-B exposure (Björn and Wang, 2001). 7-dehydrocholesterol is suitable as a radiation sensor because of the high quantum yield for photo conversion (Björn and Wang, 2001). The action spectrum for conversion of 7-dehydrocholesterol to vitamin D_3 has been determined to have a peak at 297 nm (MacLaughlin et al., 1982). Since this UV absorption spectrum completely overlaps the UV absorption spectra for DNA, RNA and proteins it is possible that 7-dehydrocholesterol evolved to protect the UV-sensitive macromolecules from UVB-exposure (Holick, 2003). Whether vitamin D in phytoplankton has a biological effect remains to be determined. However, Fries (1984) observed an increased growth of the brown algae *Fucus spiralis*, the green macroalgae *Enteromorpha compressa* and the red algae *Nemalion helminthoids* after treatment with vitamin D_2 and vitamin D_3.

9. Concluding remarks

Today, we know that vitamin D_3 and its metabolites are formed in certain plant species, but any increase in our knowledge about vitamin D in plants will be of great value. The investigation of plants as a source of vitamin D could in the future contribute to improve the vitamin D status of the general population. However, it remains to be fully established how vitamin D_3 is formed in plants. Both cholesterol and 7-dehydrocholesterol has been found in plants. It is possible that plants synthesize 7-dehydrocholesterol and subsequently vitamin D_3 through lanosterol as is known from vertebrates. However, it remains to be discovered whether vitamin D_3 in plants is formed from lanosterol and/or cycloartenol. SMT1 has been shown to interfere with the cholesterol level and by blocking SMT1 the pathway is forced in direction of 7-dehydrocholesterol and cholesterol production. This could be a tool for bio-fortification of plants to increase the amount of vitamin D_3. However, any increase in accumulation of vitamin D_3 should be viewed in the context of the overall changes in the metabolic profile e.g., in which the level of other compounds changes. Sterol biosynthesis in plants is complex and is still poorly understood and makes the final goal to produce plants with vitamin D_3 an immense challenge.

Figures and Tables

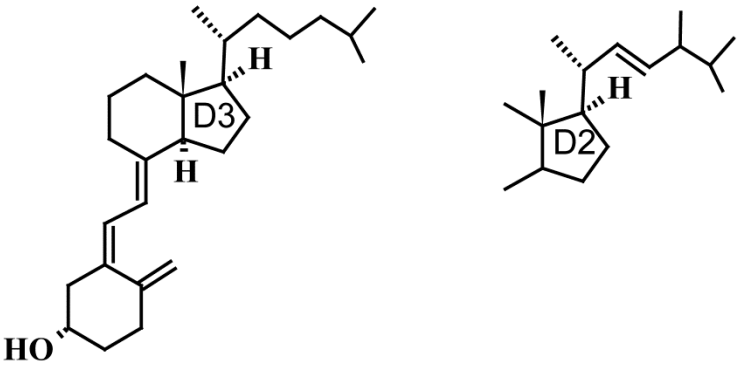

Figure 1. Structures of vitamin D_2 and vitamin D_3.

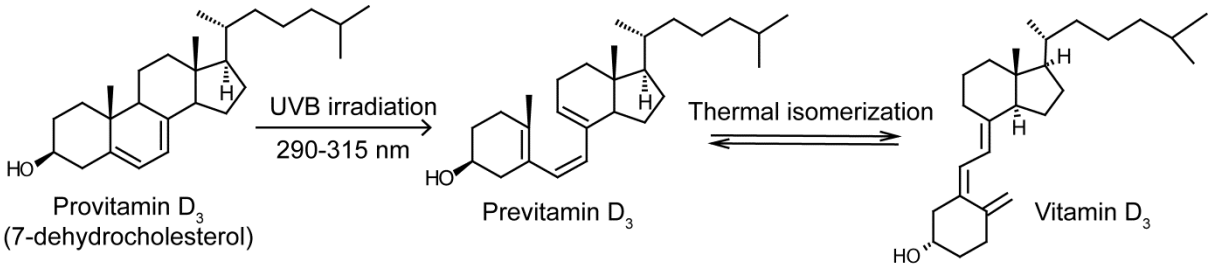

Figure 2.Biosynthesis of vitamin D_3 from 7-dehydrocholesterol. UVB exposure of provitamin D_3 (7-dehydrocholesterol) in the skin breaks the B-ring to form the previtamin D_3, which undergoes thermally induced rearrangement to vitamin D_3.

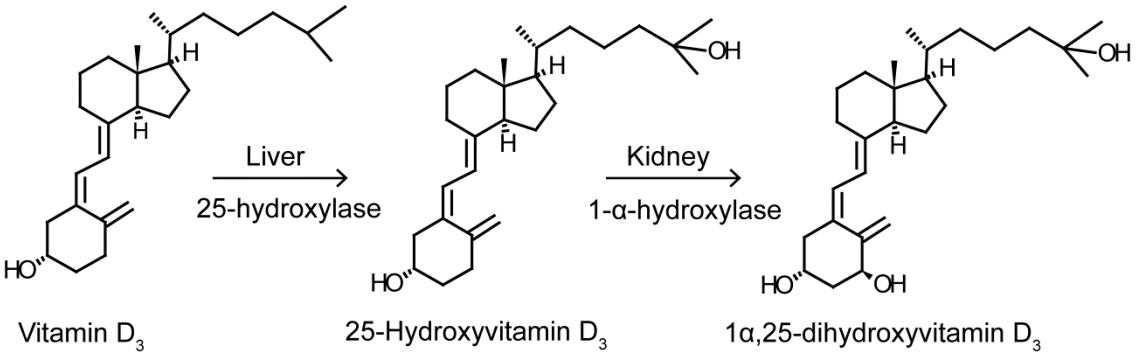

Figure 3. Vitamin D_3 is transported to the liver where it is hydroxylated at C-25 by the enzyme 25-hydroxylase producing 25-hydroxy vitamin D_3, which is the major circulating form in vertebrates. The 25-hydroxy vitamin D_3 is hydroxylated a second time at C-1 in the kidneys to produce the active metabolite 1α,25-dihydroxy vitamin D_3.

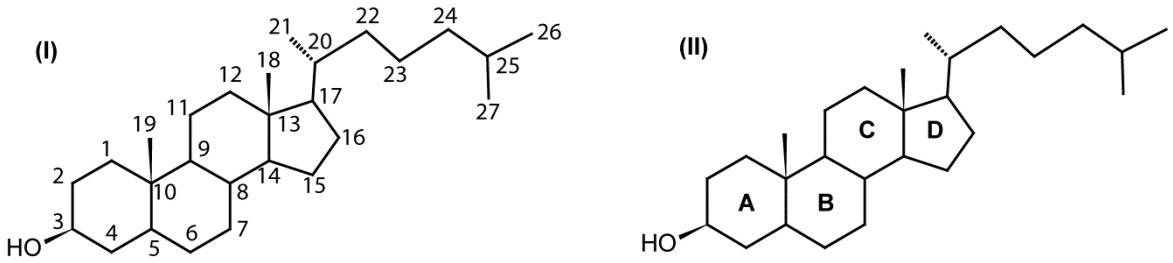

Figure 4. Sterol structures (I) with numbering of the carbon atoms and (II) with naming of the rings.

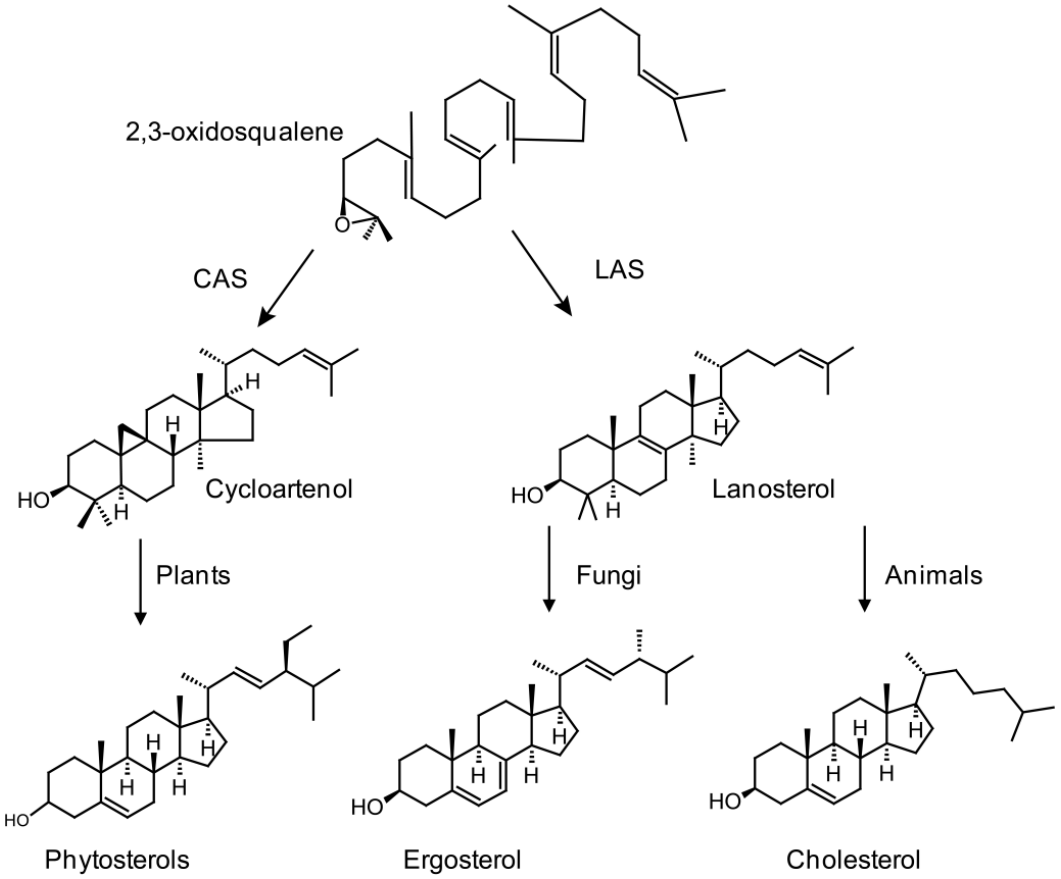

Figure 5. Cyclization of 2,3-oxidosqualene forms either lanosterol or cycloartenol via a series of enzymatic cyclizations leading to sterols in plants, fungi and animals

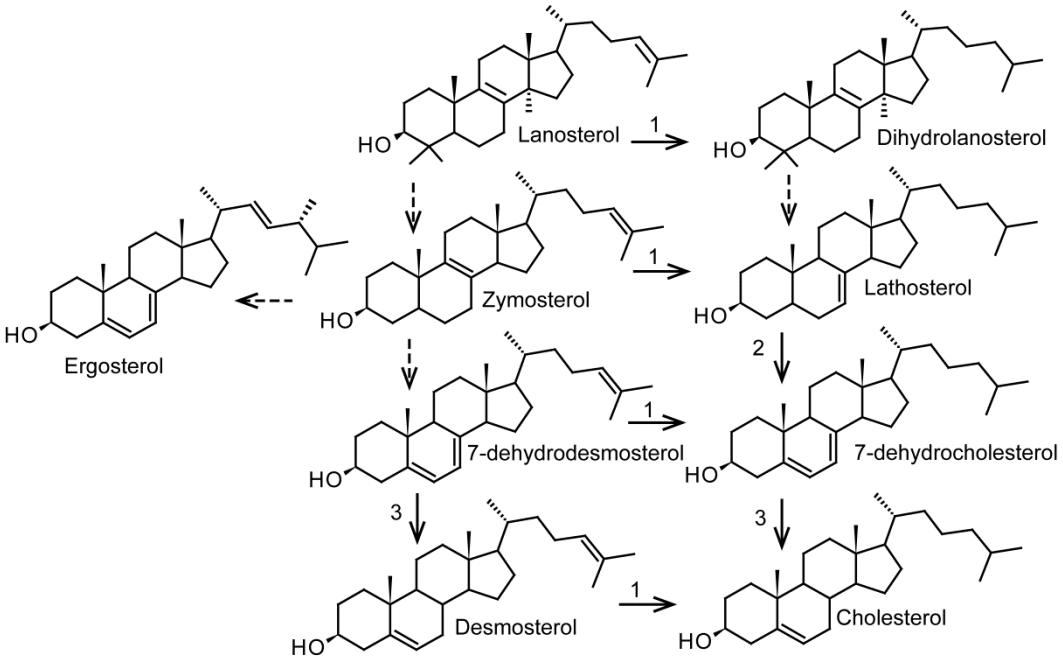

Figure 6. Simplified cholesterol and ergosterol biosynthesis pathway. Dashed arrows corresponds to multiple enzymatic reactions. Enzymes are denoted by numbers. 1: Δ^{24}-reductase, 2:lathosterol 5-desaturase, 3:Δ^{7}-reductase.

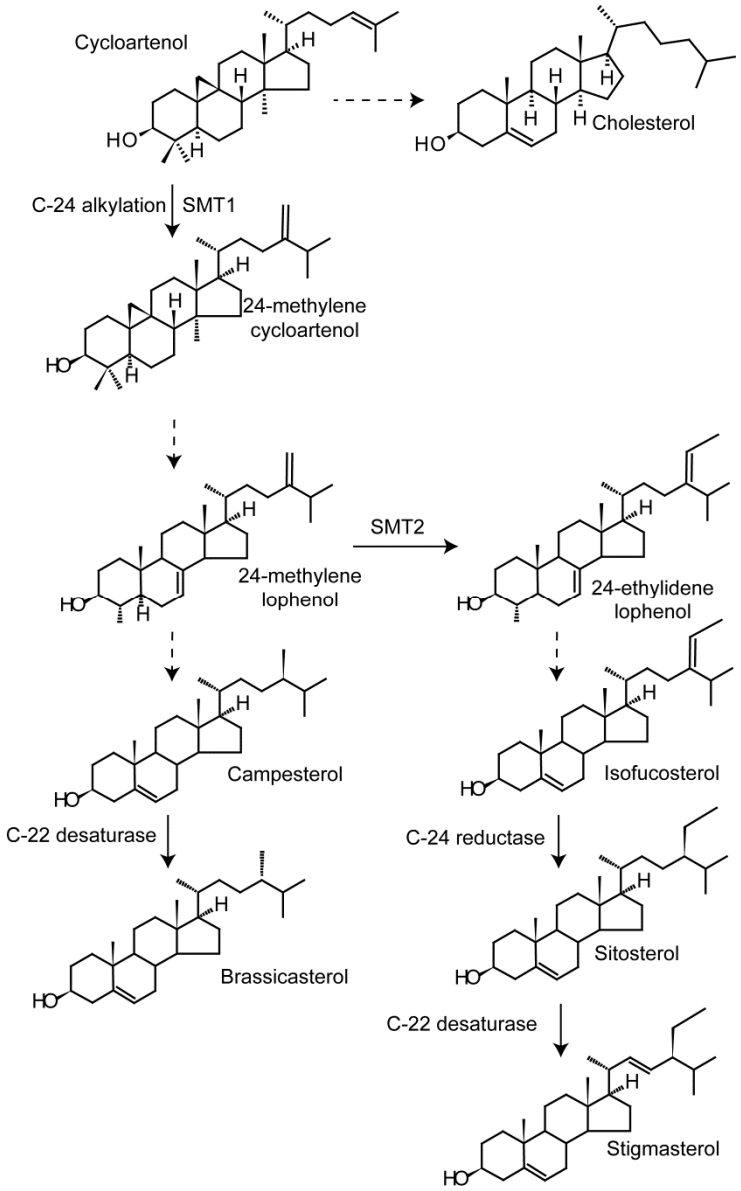

Figure 7. Simplified post-squalene biosynthetic pathway for sterols in higher plants. The dashed arrows indicate more than one biosynthetic step. The diagram represents the biosynthetic pathways of cycloartenol to 24-methyl and 24-ethyl sterols.

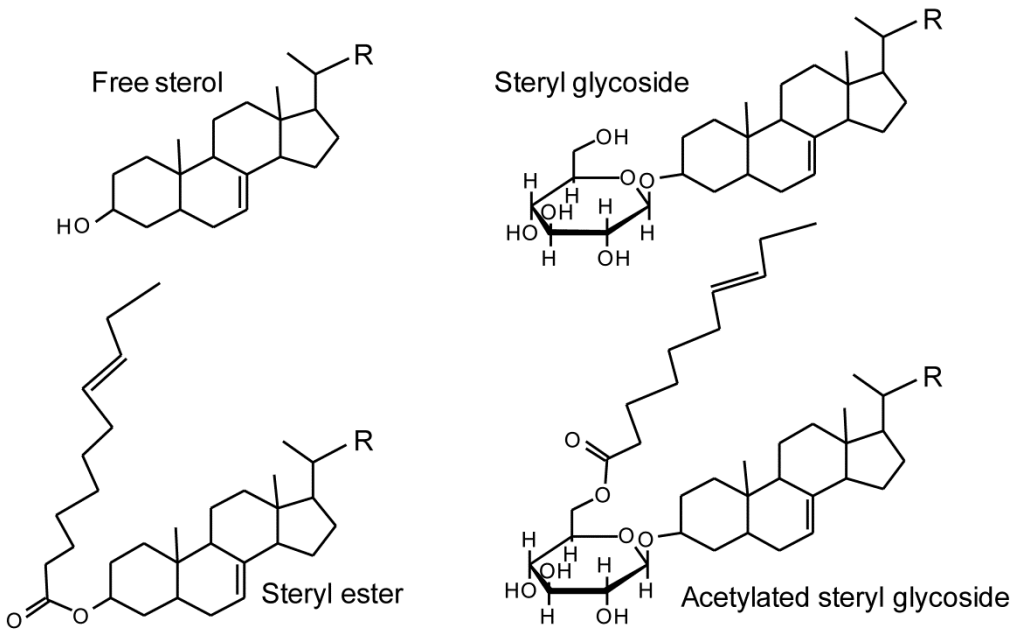

Figure 8. Basic structures of free sterol and its conjugates. The side chain R varies between sterols.

Table 1. Plants in which vitamin D_3 and 7-dehydrocholesterol have been identified by chemical methods

Species	Vitamin D_3	7-dehydrocholesterol
Solanum lycopersicum L.	0.28 µg/g dry wt.[1] 0.09 µg/g dry wt.[2] 1.1 µg/g fresh wt.[3] 0.8 ug/g dry wt.[4]	0.61-0.76 µg/g dry wt.[1] 0.23-0.47 µg/g dry wt.[2]
Solanum tuberosum L.	0.15 µg/g fresh wt.[1]	-
Cucurbita pepo L.	0.23 µg/g fresh wt.[1]	-
Solanum glaucophyllum Desf.	0.21 µg/g dry wt.[2] 2.2-42.1µg/g fresh wt.[5] Identified[6,7]	0.67-1.26 µg/g dry wt.[2] 5-58µg/g fresh wt.[5] Identified[6,7]
Nicotiana glauca Graham	Identified[8]	Identified[8]
Cestrum diurnum L.	0.1 µg/g fresh wt.[9]	-
Medicago sativa L.	0.00062-0.001 µg/g dry wt.[10]	-
Trisetum flavescens Beauv.	0.1 µg/g dry wt.[11]	-
Capsicum annuum L.	-	0.03 µg/g[2]

[1]Björn and Wang (2001), [2]Jäpelt et al. (2011b), [3]Aburjai et al. (1998), [4]Prema and Raghuramulu (1996), [5]Aburjai et al. (1996), [6]Curino et al. (2001), [7]Curino et al. (1998), [8]Skliar et al. (2000), [9]Prema and Raghuramulu (1994), [10]Horst et al. (1984), [11]Rambeck et al. (1979)

Table 2. Plants in which the hydroxylated metabolites of vitamin D_3 have been identified

Species	$25OHD_3$	$1,25(OH)_2D_3$
Solanum lycopersicum L.	0.15 µg/g fresh wt.[1]	-
	0.022 µg/g dry wt.[2]	0.10 µg/g dry wt.[2]
Solanum glaucophyllum Desf.	Identified[3,4]	Identified[3,4]
	1.0 µg/g fresh wt.[5]	0.1 µg/g fresh wt.[5]
Cestrum diurnum L.	0.102 µg/g dry wt.[6]	1 µg/g dry wt.[6]
Nicotiana glauca Graham	Identified[7]	0.3-1 µg/g fresh wt.[7]

[1]Aburjai et al. (1998), [2] Prema and Raghuramulu (1996), [3]Curino et al. (1998), [4]Esparza et al. (1982), [5]Aburjai et al. (1996), [6]Prema and Raghuramulu (1994), [7]Skliar et al. (2000)

Table 3. Content of ergosterol, vitamin D_2, 7-dehydrocholesterol and vitamin D_3 in phytoplankton, contents are displayed as µg/g dry weight

	Ergosterol	Vitamin D_2	7-dehydrocholesterol	Vitamin D_3
Japan August[1]	10.1	0.043	14.5	0.147
Japan October[1]	2.9	0.0189	3.6	0.0496
Japan December[1]	2.6	-	3.4	0.0217
India[2]	3.9	0.0525	23.6	0.803

[1]Takeuchi et al. (1991), [2]Sunita Rao and Raghuramulu (1996a)

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