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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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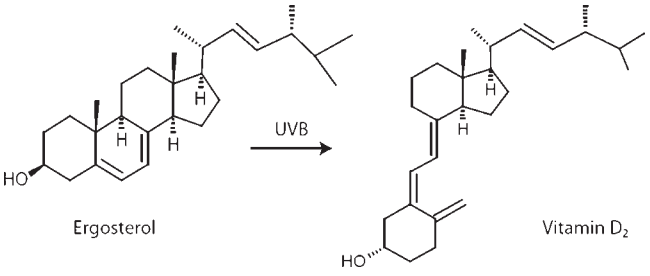

Figure 1. Conversion of provitamin D_2 (ergosterol) to vitamin D_2 by exposure of UV-B light.

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.

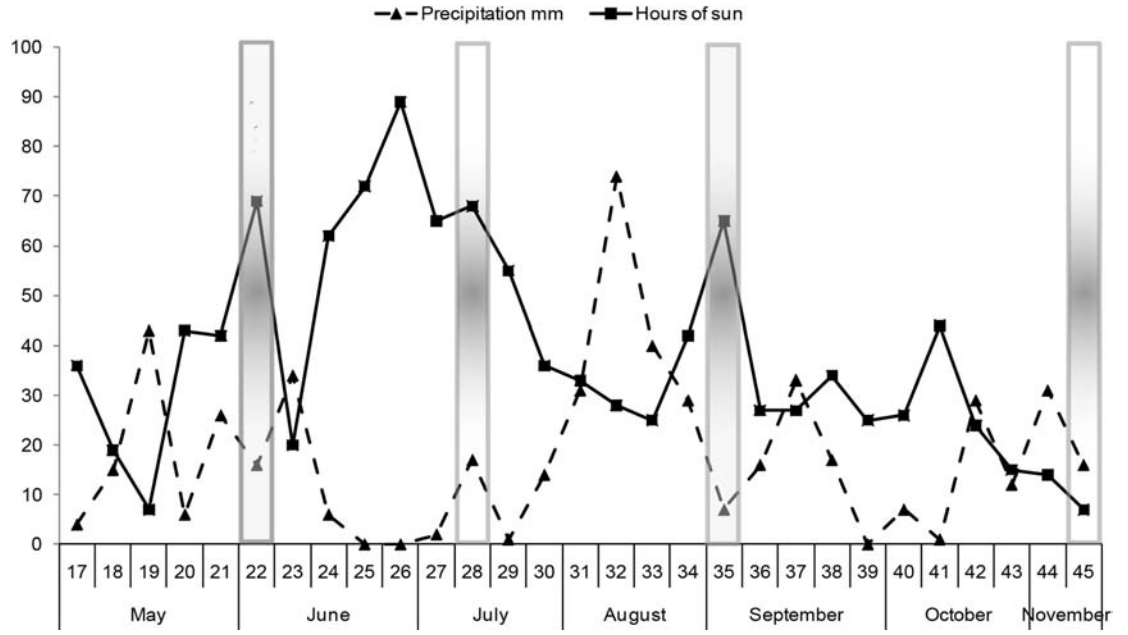

Figure 2. Precipitation (mm) and hours of sun for the period May 2010 until November 2010; the week of harvest is indicated with bars.

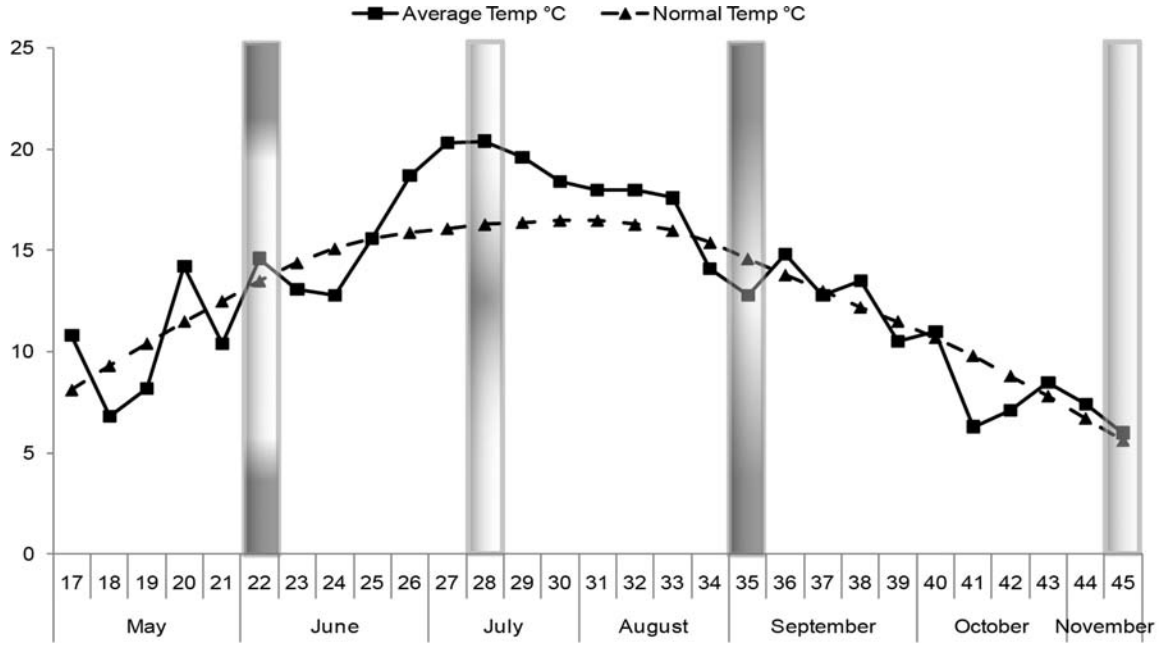

Figure 3. Normal temperature and average temperature for the period May 2010 until November 2010; the week of harvest is indicated with bars.

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

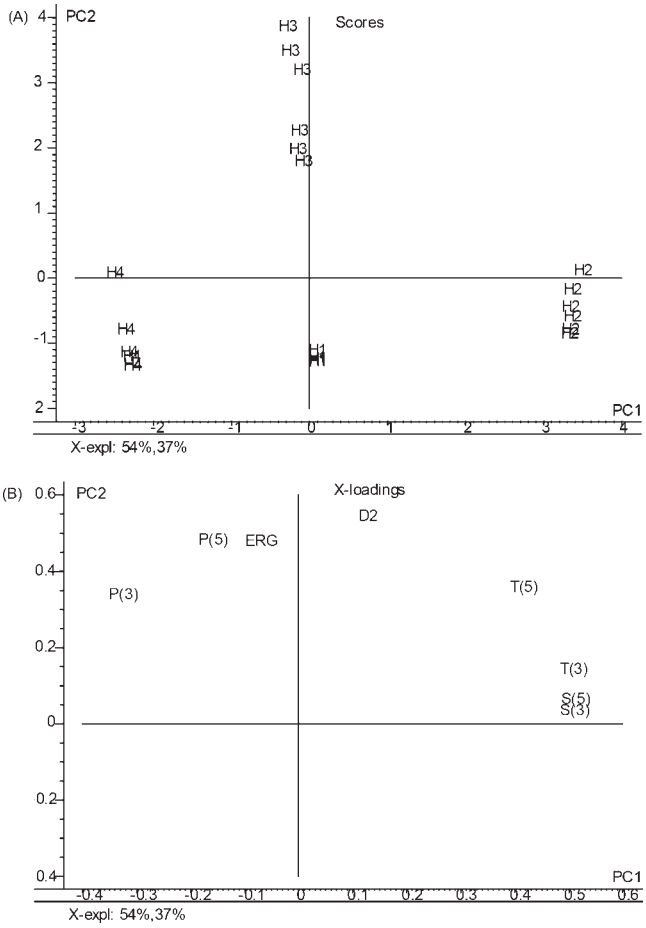

Figure 4. (A) Score plot of PC1 vs PC2, where harvest times are used as plotting symbols, H1 corresponds to the June harvest, H2 corresponds to the July harvest, H3 corresponds to the September harvest, and H4 corresponds to the November harvest. (B) Loading plot of PC1 vs PC2; variables used were vitamin 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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