Downloaded from orbit.dtu.dk on: Aug 07, 2019

DTU Library

Vitamin D Enhanced Foods Using Articial UVB Development of Products and Quantication of Vitamin D

Barnkob, Line Lundbæk

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Barnkob, L. L. (2019). Vitamin D Enhanced Foods Using Articial UVB Development of Products and Quantication of Vitamin D. Kgs. Lyngby: Technical University of Denmark.

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

DTU Food
National Food Institute

Vitamin D Enhanced Foods Using Artificial UVB

Development of Products and Quantification of Vitamin D

Line Lundbæk Barnkob
PhD Thesis
2019

Vitamin D Enhanced Foods Using Artificial UVB

Development of products and quantification of vitamin D

PhD thesis

Line Lundbæk Barnkob

Research Group for Bioactives – Analysis and Application
National Food Institute
Technical University of Denmark

2019

Vitamin D Enhanced Foods using Artificial UVB
Development of products and quantification of vitamin D

PhD thesis
2019

Kgs. Lyngby

Copyright: Reproduction of this publication in whole or in part must include the customary bibliographic citation, including author attribution, report title, etc.
Cover photo: Colourbox.com
Published by: National Food Institute, Kemitorvet, Building 202, 2800 Kgs. Lyngby Denmark
Available at: www.food.dtu.dk

Preface

This thesis was submitted as partial requirement for obtaining my Doctor of Philosophy degree at the Technical University of Denmark. The research was conducted from 2013 to 2018 (including 2 maternity leaves) at DTU Food, National Food Institute. A grant from the European Commission under its Seventh Framework Programme (ODIN; grant agreement no. 18 613977) and the Technical University of Denmark funded the project.

Several people contributed to the completion of this thesis. I wish to express my appreciation and gratitude to the following:

First of all I would like to thank Jette Jakobsen, my supervisor, for her guidance, understanding and support; and for her personal involvement in this project.
Kirsten Pinndal for introducing me to the analysis of vitamin D. Heidi Jahn for the many hours of pleasant company in the lab and assistance during sample collection, sample preparation, analysis of the pig samples and especially for determining the fat content. Prof. Paul Michael Petersen and Aikaterini Argyraki from DTU Fotonik for sharing their expertise and time. Anne Ørngreen and her staff from the animal facilities for taking good care of the hens and for collection of eggs. The staff at Rørrende gård for taking good care of the pigs. Henrik Frandsen for helping me put theory into practice in regards of the post-column infusion experiment. Hedegaard for supplying the hens and feed and for sharing their experience in how to care for them. Simone Santos Faria for assisting in the analysis of the eggs. My colleagues at DTU Food, who have helped me on daily basis and provided a good working environment.

Finally, I would like to thank my friends and family for always supporting me; and a special thanks to my two little girls, you are my greatest achievement.

Kgs. Lyngby, April 2019

Line Lundbæk Barnkob

Summary

It is estimated that on annual basis 40 % of the European population is vitamin D insufficient/deficient. The recommended intake of vitamin D is between 10 and 20 µg/day; however the estimated intake is 3 to 7 µg/day. A way to increase the vitamin D intake is to fortify a broader range of foods or by increasing the natural vitamin D content in food sources that already contain vitamin D. Like humans, hens and pigs can produce vitamin D when their skin is exposed to UVB.
Preliminary investigations (*ex vivo*) showed that the optimal wavelenght for vitamin D production in skin was 296 nm; and that the vitamin D_3 production depended on the dose and not the exposure time or total irradiance.
Hens and pigs were exposed to artificial UVB in order to increase the vitamin D content in eggs and pork, respectively. It was shown that the vitamin D content in eggs could be increased when the hens were exposed daily to ≥ 4000 J/m^2 UVB (306.5 nm) from above, however due to erythemal change of the comb the trial was stopped after 7 days. Based on existing published data a linear response between vitamin D_3 in feed and vitamin D_3 content in eggs was shown. It was concluded that feeding higher levels of vitamin D_3 to laying hens is more efficient than UVB exposure to increase the vitamin D content in eggs as it only requires a change in the feed composition. UVB emitting fluorescent tubes were installed in settings comparable to conventional pig farms. With a dose of 1 SED/day for 28 days (including 7 days adaption period) before slaughter the total vitamin D_3 content in lean meat and subcutaneous fat was a factor 19 and 29 higher, respectively, compared to the control; while $25(OH)D_3$ was a factor 8 higher in both compared to the control.
It was shown possible to produce vitamin D enhanced pork rind and liquid egg products (whole eggs and yolk) by direct exposure to UVB. The vitamin D content of such products could be tailored by adjusting the dose of UVB.
All methods presented still calls for further research and approval in the EU before it can be implemented in industry.
Continuous post-column infusion of vitamin D_3-d_6 was used to visualise ion suppression in the analysis of vitamin D_3 as no blank matrix was available; by using this method it was possible to visualise the effect different eluent additives had on ion suppression in extracts of different food matrices. With a sample preparation consisting

of saponification, liquid-liquid extraction, SPE and derivatisation with PTAD an enormous amount of ion suppression of vitamin D_3 was observed. Due to its earlier retention time vitamin D_3-d_6 could not fully eliminate matrix effects in most food matrices.

Resumé (in Danish)

På årsbasis har omkring 40 % af den Europæiske befolkning enten D-vitamin insufficiens eller mangel. Det anbefalede daglige indtag af D-vitamin ligger mellem 10 og 20 µg, men det estimerede indtag er omkring 3-7 µg/dag. Det daglige indtag kan øges ved at berige en bredere vifte af fødevarer eller øge det naturlige indhold af D-vitamin indhold i fødevarer. Høns og grise kan ligesom mennesker producere D-vitamin i huden hvis de bliver eksponeret til UVB-lys.

Indledende forsøg (*ex vivo*) viste at den optimale bølgelængde for produktion af D_3-vitamin i hud var 296 nm, og at produktionen afhænger af dosis og ikke af eksponeringstiden eller bestrålingsstyrken.

Høns og grise blev belyst med kunstigt UVB-lys med det formål at øge indholdet af D-vitamin i æg og svinekød. Indholdet i æg kunne øges ved at eksponere høns til doser ≥ 4000 J/m^2 UVB-lys/dag (306.5 nm) ovenfra, men forsøget blev stoppet efter 7 dage da der blev observeret ændringer på kammene af hønsene. Ud fra publiceret data blev der fundet en lineær sammenhæng mellem D_3-vitamin i foder og D_3-vitamin indholdet i æg. Konklusionen blev at fodring af høns med højere niveauer af D_3-vitamin er en mere effektiv måde at øge indholdet af D_3-vitamin i æg på, end at belyse hønsene med UVB-lys, da det kun kræver en ændring i fodersammensætningen.

UVB lysstofrør blev installeret i en svinestald. I de sidste 28 dage inden slagtning blev grise belyst med en 1 SED per dag (inkl. 7 dages tilvænningsperiode). I forhold til kontrolgruppen var indholdet af D_3-vitamin i svinefilet og subkutant fed henholdsvis 19 og 29 gange højere og 25-hydroxyvitamin D_3 indholdet var en faktor 8 højere i begge.

D-vitamin indholdet kunne øges i svær fra grise og flydende æggeprodukter (hele æg og blommer) ved at belyse produkterne direkte med UVB-lys. D-vitamin indholdet i sådanne produkter kan tilpasses ved at justere den anvendte dosis af UVB-lys.

Samtlige af de præsenterede metoder kræver yderligere forskning og godkendelse i EU for de er klar til at blive implementeret i industrien.

Hvordan forskellige løbevæske additiver påvirker ioniseringen af D_3-vitamin i ekstrakter fra forskellige fødevarer ved LC-ESI-MS/MS analyse blev visualiseret ved brug af continuous post-column infusion af D_3-d_6-vitamin da det ikke var muligt at skaffe fødevarematricer (der er kilde til D-vitamin) uden D-vitamin. Efter en prøveforberedelse bestående af forsæbning, væske-væske-ekstraktion, fastfase-ekstraktion og

derivatisering med PTAD viste ionsuppressionen af D_3-vitamin sig at være meget omfattende. Da D_3-d_6-vitamin eluere lidt før D_3-vitamin kan brugen af D_3-d_6-vitamin ikke gøre op for matrix effekten fra størstedelen af de undersøgte fødevarematricer.

List of publications

Paper I: **Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin**
Line Lundbæk Barnkob, Aikaterini Argyraki, Paul Michael Petersen and Jette Jakobsen
Food Chemistry, 2016; 212; 386-391.

Paper II: **Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities**
Line Lundbæk Barnkob, Paul Michael Petersen, Jens Peter Nielsen and Jette Jakobsen
European Food Research and Technology, 2018; 245(2); 311-418.

Paper III: **Naturally enhanced eggs as a source of vitamin D: A review**
Line Lundbæk Barnkob, Aikaterini Argyraki and Jette Jakobsen
Submitted to Trends in Food Science and Technology

Paper IV: **Deuterated internal standard in the analysis of vitamin D in foods does not always correct for matrix effects: the effect of eluent additives**
Line Lundbæk Barnkob, Henrik Lauritz Frandsen and Jette Jakobsen
Prepared for submission to Journal of Chromatography A

List of abbreviations

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
7-DHC	7-dehydrocholesterol
DBP	Vitamin D binding protein
EFSA	European Food Safety Authority
ESI	Electrospray ionization
L_2	$Lumisterol_2$
L_3	$Lumistrol_3$
LC-MS/MS	High performance liquid chromatography tandem mass spectrometer
LED	UV light-emitting diodes
ME	Matrix effect
MRM	Multiple reaction monitoring
MS/MS	tandem mass spectrometer
NRC	National Research Council
$PreD_3$	Previtamin D_3
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
RT	Retention time
RTC	Randomised controlled trial
SIL IS	Stable isotope labelled internal standard
SRM	Selected reaction monitoring
T_3	$Tachysterol_3$
UV	Ultraviolet
UVB	Ultraviolet B
WP 5	Work package 5

Content

1. Introduction 1

2. Background 5
2.1 Vitamin D and health 5
2.2 Sources of vitamin D 6
2.3 Endogenous synthesis of vitamin D 6
2.4 Absorption of dietary vitamin D 7
2.5 Metabolism of vitamin D 8
2.6 Photodegradation of vitamin D 9
2.7 PreD-vitamin D equilibrium 11
2.8 Biological activity of the vitamin D vitamers 13

3. Quantification of vitamin D in food 15
3.1 Theory behind ESI-MS/MS 15
3.1.1 ESI 15
3.1.2 MS/MS 16
3.2 Saponification – an essential step in the analysis of vitamin D 17
3.3 Analytical methods used in the project 18
3.3.1 Effect of changing the eluent additive on the quantification of vitamin D_3 19
3.4 Investigating the effect of different eluent additives in vitamin D_3 analysis (**Paper IV**) 20
3.4.1 Deuterated SIL IS does not always correct for matrix effects 20
3.4.2 Determination of matrix effects 21
3.4.3 Continuous post-column infusion of vitamin D_3-d_6 to visualise ion suppression 21
3.4.4 Effect of eluent additives on adduct formation 24
3.5 Discussion of results and future perspectives for improvement of the analytical method 25

4. Artificial UVB for vitamin D production 27
4.1 Choice of UVB source 27
4.2 The optimal wavelength for vitamin D production in skin (**Paper I**) 28
4.3 Effect of dose (Paper I) 31
4.4 Influence of exposure time and total irradiance (**Paper I**) 32

5. Vitamin D enhanced foods using artificial UVB 35
5.1 Vitamin D enhanced eggs 35
5.1.1 UVB irradiation of hens from above 35
5.1.1.1 Animals and test environment 35
5.1.1.2 Description of UVB lamps used in the hen trials 37
5.1.1.3 Sample preparation and analysis 39
5.1.1.4 Vitamin D content in eggs at the start of the trial 40
5.1.1.5 Trial 1 40
5.1.1.6 Trial 2 43
5.1.1.7 Trial 3 44
5.1.2 Direct UVB irradiation of liquid egg products 45
5.2 Vitamin D enhanced pork products 46

5.2.1 Description of the UVB emitting fluorescent tubes used in the pig trial **(Paper II)** 46
5.2.2 UVB irradiation of pigs (**Paper II**) 48
5.3 Discussion..54
5.3.1 Choice of UVB source 54
5.3.2 Effectiveness of UVB exposure compared to feeding vitamin D for the enhancement of eggs (**Paper III**)..55
5.3.2.1 European legislation regarding vitamin D in feed for hens.......................................57
5.3.3 Possible dual effect of UVB when used for direct exposure of liquid egg products 58
5.3.4 Novel foods regulation in the EU 58

6. Conclusion **59**

7. Perspectives **61**

References...62

Publications...71

1. Introduction

Sun exposure is the major contributor to vitamin D in humans. The precursor of vitamin D_3, 7-dehydrocholesterol (7-DHC) is located in the skin. When skin is exposed to light that includes ultraviolet B rays (UVB, 280-315 nm) 7-DHC is converted to previtamin D_3 (preD_3) that subsequently is converted to vitamin D_3. Vitamin D_3 is converted to its major circulating metabolite 25-hydroxyvitamin D_3 (25(OH)D_3) in the liver; and currently the serum 25(OH)D level is the generally accepted biomarker for vitamin D status. The ozone layer effectively attenuates UVB especially at lower wavelengths with the result that wavelengths ≤ 290 nm does not reach the surface of the earth; and due to the tilt of the earth, the solar photos have a longer travel through the atmosphere during winter and thereby higher probability of attenuation (Engelsen 2010; Webb 2006). For this reason no cutaneous vitamin D_3 can be produced at least part of the winter if above or below 35 °N and 35 °S, respectively; at 52 °N the "vitamin D winter" stretches from October through March (Webb et al. 1988; O'Neill et al. 2016). During winter we therefore rely on food as our main source of vitamin D and the recommended intake is between 10 and 20 µg/day (Institute of Medicine 2011; Nordic Council of Ministers 2012). The daily intake in Europe is however estimated to be around 3-7 µg/day (Cashman & Kiely 2016). The consequence is that on annual basis around 13 % of the European population have a vitamin D status below 30 nmol/L and 40 % a vitamin D status below 50 nmol/L (Cashman et al. 2016) where ≤ 50 nmol is considered insufficient and ≤ 30 nmol/L is considered deficient (Nordic Council of Ministers 2012). The intake can be increased using food-based strategies (O'Mahony et al. 2011; Black et al. 2012); one such strategy is to fortify a broader range foods and/or increase the natural content of vitamin D in foods (Cashman & Kiely 2016; Kiely & Black 2012). Like humans livestock can obtain vitamin D either by endogenous production when exposed to UVB (Burild et al. 2015; Jakobsen et al. 2015; Schutkowski et al. 2013; Larson-Meyer et al. 2017; Kolp et al. 2017; Alexander et al. 2017) or through feed (Jakobsen et al. 2007; Burild et al. 2016; Mattila et al. 2004); either way the vitamin D content in the animal products will be increased. Food products that contain 7-DHC can also be enhanced with vitamin D by exposing the product directly to UVB, so far mushrooms, milk and baker's yeast have been enhanced using this method (Kristensen et al. 2012; O'Brien et al. 1938; EFSA NDA Panel 2014). 7-DHC is also present in pig skin (Burild et al. 2015) and in egg yolk (Kühn et al. 2014).

Based on this the first two hypotheses of this thesis were:

1. That eggs and pigs in large scale productions can be enhanced with vitamin D by irradiation with UV-light using methods that are applicable in an industrial food production.

2. That vitamin D enhanced food products can be produced by exposing pig rind or liquid egg products directly to UVB.

When analysing compounds using liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS) there is a risk that other compounds present in a sample will either enhance or suppress the ionisation of the compound – this is known as the matrix effect (ME). If a pure standard, of the compound of interest, and a sample, without it, is available it will be possible to determine the ME by a continuous post-column infusion experiment (Bonfiglio et al. 1999); this will give an imprint of all MEs present in the sample. However, when analysing inherent compounds in food stuff, e.g. vitamin D, it is not possible to get a blank sample. For this reason the determination of ME on inherent compounds has so far been based on recovery methods.

Based on this the third hypothesis was:

3. That stable isotope labelled internal standard compounds (SIL IS) can be used to determine matrix effects by continuous post-column infusion experiments with the point of visualising how eluent additives affect the ionisation of vitamin D_3.

The thesis includes two published articles (**Paper I** and **Paper II**), one submitted manuscript (**Paper III**) and a manuscript prepared for submission (**Paper IV**).

The structure of the thesis is as follows:
Background information is reviewed in Chapter 2. In Chapter 3 the different analytical methods used to determine vitamin D throughout the project is presented and some critical issues relating to the analysis of vitamin D are discussed and the results of the continuous post-column infusion experiment is presented (**Paper IV**). Based on the experiments with direct UVB exposure of pig skin (**Paper I**) the use of artificial UVB for

vitamin D production is presented in Chapter 4. The results from UVB exposure of hens and pigs and direct exposure of egg yolk are found in Chapter 5 (**Paper II** and **Paper III**). The conclusion and perspectives are presented in Chapter 6 and 7, respectively.

2. Background

Chapter 2 gives the necessary background information in regards to vitamin D. The focus is on different aspects of vitamin D in the human body such as the endogenous synthesis, absorption of dietary vitamin D, its metabolism, the biological activity of the metabolites and the effect of vitamin D on the health. The conversion of preD_3 to vitamin D_3 in skin and solvent systems and how they differ is also included.

2.1 Vitamin D and health

Vitamin D is vital for the maintenance and regulation of calcium levels in vertebrates. Its primary function is to increase the calcium uptake (and possible also phosphate) in the intestines. It also promotes renal reabsorption of calcium (Christakos et al. 2016). More than 99 % of calcium in the body is stored in the bones and teeth and together with phosphor it makes up the mineral structure of the skeleton (Institute of Medicine 1997). During prolonged periods of vitamin D deficiency the calcium uptake will be too low to support bone mineralisation; this will lead to softening of bones in children, a condition known as rickets, and osteomalacia in adults where the bones become more fragile (Underwood & DeLuca 1984).

However, vitamin D does not only affect bone health. The expression of up to 5 % of the human genome may be affected by vitamin D. A range of observational studies have found association between low vitamin D status and a large number of diseases such as cancer, cardiovascular diseases, diabetes, obesity, infections and autoimmune diseases. However, so far no final proof of the effect of vitamin D on such other diseases has been published (reviewed by Rejnmark et al. (2017)).

The level of sufficiency was traditionally based on bone health, however one might have sufficient vitamin D to prevent rickets, but still be deficient with respect to other endpoints (Heaney et al. 2009). On annual basis 40 % of the European population have an vitamin D status below 50 nmol/L (Cashman et al. 2016), which is considered insufficient (Nordic Council of Ministers 2012); the vitamin D intake should therefore be increased (Pilz et al. 2018).

2.2 Sources of vitamin D

With a whole range of reservations regarding individual behaviour, ethnicity and latitude it is roughly estimated that sun exposure contributes with 80 % of the total vitamin D in healthy humans (Pilz et al. 2018). Food has a minor contribution in winter time (above latitude 50 °), and the contribution is insignificant during summer (Poskitt et al. 1979; Lawson et al. 1979; Andersen et al. 2013). Vitamin D is naturally present in very few food sources where fish and eggs are the main contributors and minor content is found in meat and dairy products (Ovesen et al. 2003).

Vitamin D_3 comes from animal sources while vitamin D_2 is produced in fungi exposed to UVB; however, the metabolism in the human body of the two is similar.

2.3 Endogenous synthesis of vitamin D

When 7-DHC, present in the skin, is exposed to UVB the energy in the photons is absorbed by the conjugated diene in the B ring. The excess of energy will cause the B ring to open between carbon number 9 and 10 whereby pre-vitamin D_3 ($PreD_3$) is produced. $PreD_3$ is thermally isomerized, by a rearrangement of the triene system, to vitamin D_3, as shown in

Figure 2-1. The equilibration between $PreD_3$ and vitamin D_3 favours the formation of vitamin D_3. Vitamin D_3 is actively transported from the skin into circulation by binding to vitamin D binding protein (DBP) in the capillary bed of the skin (Holick et al. 1980). Although 7-DHC is present both in epidermis and dermis the main production of $preD_3$ is in the *stratum spinosum* and *stratum basale* which is the two inner layers of the epidermis (Holick et al. 1980).

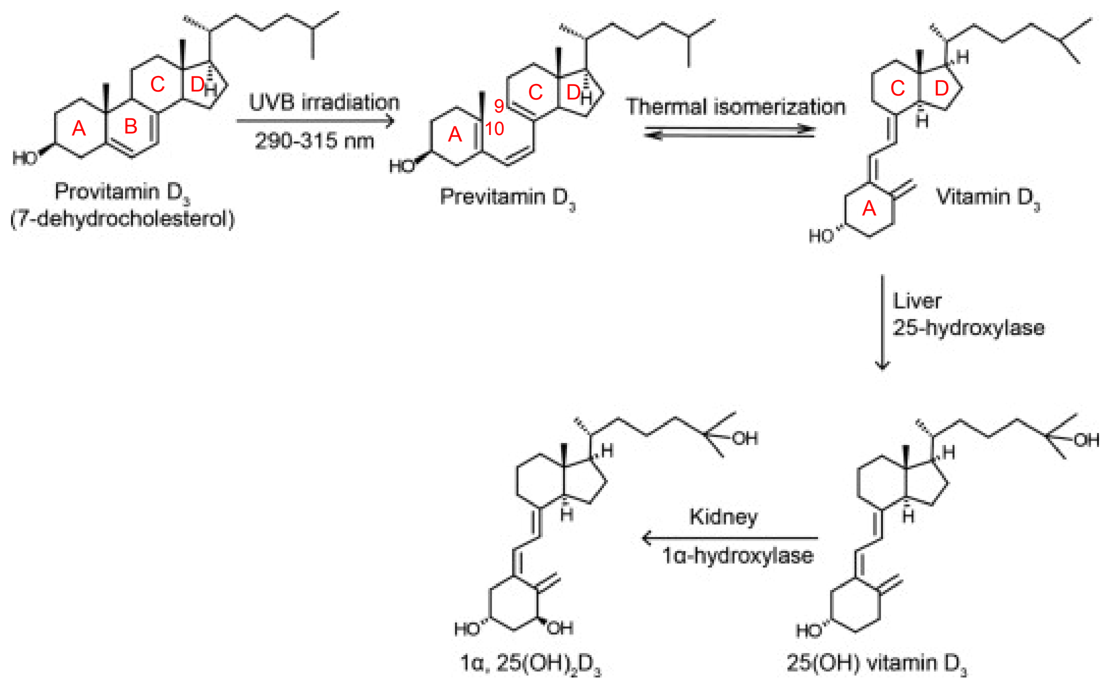


Figure 2-1 The photo production and activation of vitamin D_3. (From Jäpelt et al. (2013) with permission, modified)

Not only humans produce vitamin D_3 from 7-DHC in the skin; e.g. cows, pigs, rats and hens can utilize this mechanism as well (Luce 1924; Steenbock et al. 1924; Uva et al. 1983; Maughan 1928; Okano et al. 1978). The conversion of 7-DHC to preD_3 is a physical process and can therefore proceed in dead skin tissue and in foods containing 7-DHC (e.g. milk) as well (O'Brien et al. 1938; Okano et al. 1978).

2.4 Absorption of dietary vitamin D

Dietary vitamin D is incorporated into mixed micelles in the stomach and duodenum and the major uptake happens in the jejunum and ileum. The micelles can bind to at least three specific transporter proteins on the enterocytes, and thereby the content is selectively released into the cell. These transporter proteins also have affinity for other lipid compounds such as cholesterol, vitamin K and vitamin E. In the endoplasmic reticulum vitamin D_3 is incorporated into chylomicrons without modification. The chylomicrons are subsequently released into the mesenteric lymph duct. Vitamin D can also be absorbed by passive diffusion however this mainly occurs at pharmacological doses (Reviewed by Reboul (2015)). Significant amounts of the vitamin D contained in the chylomicrons are transferred to DBP and then to the blood circulation (Blomhoff et al. 1984; Dueland, Pedersen, et al. 1983; Dueland, Helgerud, et al. 1983) Vitamin D

transported in chylomicrons can also be stored in adipose and muscle tissue and what is left of vitamin D in the chylomicrons remnants is transported to the liver (reviewed by Jones (2008)).
25(OH)D absorption is independent of chylomicron formation and is mainly transferred to the mesenteric veins where it is bound to DBP (Reviewed by Reboul (2015); (Dueland, Pedersen, et al. 1983)).

2.5 Metabolism of vitamin D

An overview of the metabolism of vitamin D is displayed in Figure 2-2, while it is shown as chemical structures in Figure 2-1. No matter the source vitamin D ends up in the liver where it is hydroxylated to 25(OH)D by enzymes with 25-hydroxylase activity. Bound to DBP 25(OH)D is then transported to the kidneys where it is once more hydroxylated this time to the active metabolite 1,25$(OH)_2$D by enzymes with 1α-hydroxylase activity. Enzymes with 1α-hydroxylase activity has also been found in extra-renal tissue (reviewed by Christakos et al. (2016)). 25(OH)D is the major circulating metabolite and its production is relatively unregulated while the production of 1,25$(OH)_2$D is very tightly regulated; its production is stimulated by the parathyroid hormone (PTH) and inhibited by fibroblast growth factor-23 (FGF-23) (reviewed by DeLuca (2004) and Christakos et al. (2016);(Pilz et al. 2018)). Vitamin D and 1,25$(OH)_2$D have approximately the same affinity for DBP, however 25(OH)D have 10-20 times higher affinity. The plasma half-life of vitamin D, 25(OH)D and 1,25$(OH)_2$D in plasma is 4-6 hours, 15 days and 10-20 hours, respectively (reviewed by Jones (2008)). The serum concentration of 25(OH)D is used as biological marker for vitamin D status as its it is the major metabolite, it has the most stable plasma concentration and it is negatively correlated to PTH which is also a marker for vitamin D status (Heaney 2014; Pilz et al. 2018). 1,25$(OH)_2$D is the only active form of the vitamin and it is strictly speaking a hormone that can bind to the vitamin D receptor (VDR) present in the cell wall of receptor cells located throughout the body in a wide variation of tissue (reviewed by DeLuca (2004) and Bikle (2016)); 1,25$(OH)_2$D can thereby influence many processes in the body as described in Section 2.1. 25(OH)D and 1,25$(OH)_2$D can be catabolised in the liver to a wide variety of inactive metabolites that eventually will be excreted; the major ones being 24,25-hydroxyvitamin D and 1,24,25-hydroxyvitamin D (reviewed by Christakos et al. (2016); (Pilz et al. 2018)).

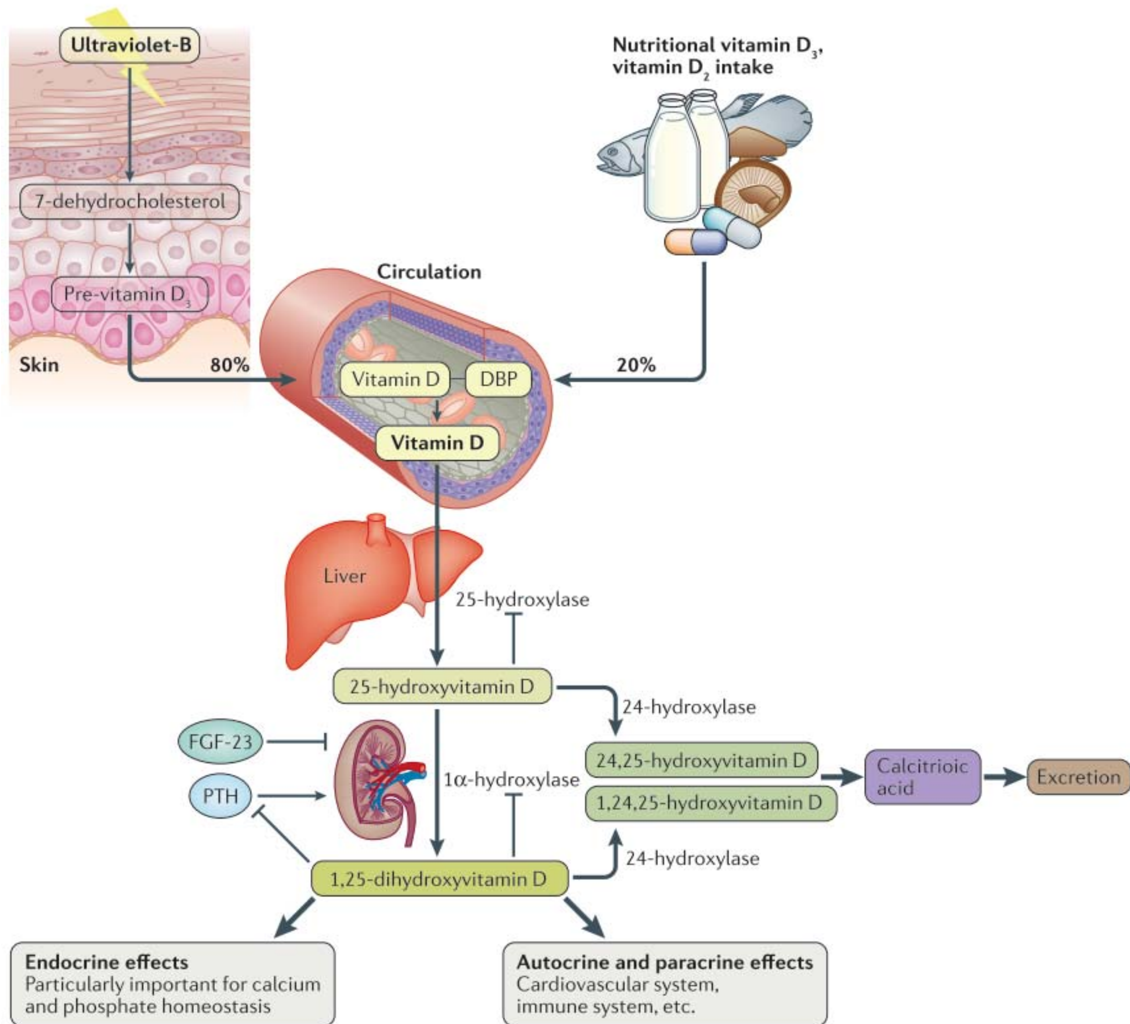


Figure 2-2 Schematic overview of sources of vitamin D and its metabolism ,function and excretion in the human body. From Pilz et al. (2016) with permission.

2.6 Photodegradation of vitamin D

As shown in Figure 2-3, UV is not only responsible for the production of $preD_3$ at prolonged exposures it also turns $PreD_3$ into $lumisterol_3$ (L_3), $tachysterol_3$ (T_3) and toxisterols and vitamin D_3 into suprasterol I and II and 5,6-transvitamin D_3 (Webb et al. 1989; Holick et al. 1981).

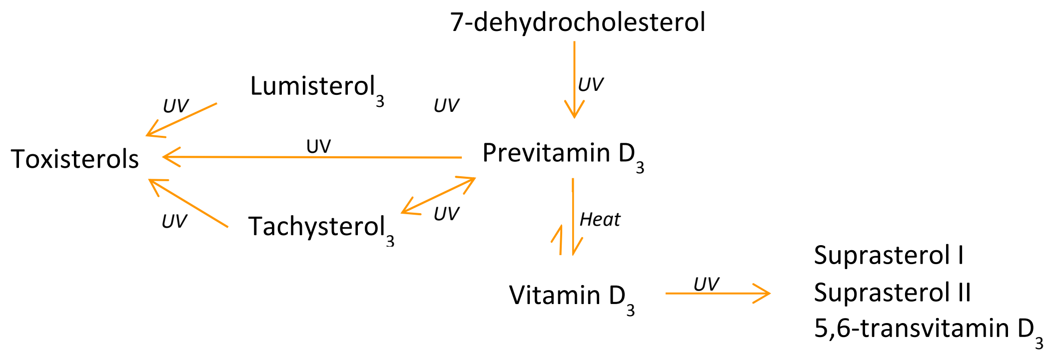


Figure 2-3 Photoisomerisation of previtamin D_3 and vitamin D_3 in the skin.

One function of the photoisomers is to avoid vitamin D intoxication; however, they have been shown to have other functions as well. *In vitro* experiments with keratinocytes (skin cells) have shown that L_3, T_3, 5,6-transvitamin D_3 and their hydroxylated metabolites have anti-proliferative activity (protects against cancer) (Chen et al. 2000; Slominski et al. 2017); L_3 and its hydroxylated metabolites have been determined in human serum (Slominski et al. 2017). L_3 is not expected to have any significant anti-rachitic activity as L_2 has 5000 times less anti-rachitic activity compared to vitamin D_2 (Bekemeier & Pfordte 1963).T_3 can in theory be isomerized back to PreD_3 at wavelengths above 320 nm and it is hypothesized that it can serve as a source of PreD_3 during winter and in the morning/evening during summer (Cisneros et al. 2017). The fate and function of toxisterols are so far unknown (Cisneros et al. 2017). Estimating the extent of photoisomerisation in living tissue is not straight forward as preD_3 is continuously converted into vitamin D_3, which is removed from the skin, therefore, equilibrium of the photoproducts are never reached, making it difficult to determine the importance of the different photoreactions (Yamamoto & Borch 1985; Holick et al. 1981). To make things more complicated the ultraviolet absorption spectrum of 7-DHC and the main photoproducts differs; therefore the amount of each photoisomer produced also depends on the wavelength (Havinga 1960; MacLaughlin et al. 1982). The main photoisomer is T_3 When 7-DHC is exposed to the a broadband spectrum of UVB while L_3 is the major when only using wavelengths ≤ 300 nm (Kühn et al. 2015; Holick et al. 1981; MacLaughlin et al. 1982; Yamamoto & Borch 1985; Dauben & Phillips 1982; Braun et al. 1991). The amount of photoisomers produced is dependent on the exposure time, the dose and previous UV exposures as well (Havinga 1960; MacLaughlin et al. 1982; Van Dijk et al. 2016). When surgically removed human skin is exposed to maximum 15 minutes of UVB similar to equatorial

spectrum and intensity the main photoproduct is $PreD_3$; during prolonged exposure $preD_3$ is photo isomerized to L_3 and T_3 (Holick et al. 1981).

2.7 PreD-vitamin D equilibrium

The reaction rate constant of the isomerisation of $preD_3$ to D_3 (k_1) is much higher than the reaction rate constant for $D_3 \leftrightarrow preD_3$ (k_2) therefore at equilibrium vitamin D_3 is the predominant isomer. k_1 and k_2 depends on temperature and they are extremely slow at low temperatures; increasing the temperature increases both (although k_2 is more sensitive to temperature) (Hanewald et al. 1961).
The conversion of $preD_3$ to vitamin D is an exothermic reaction which means that the reaction releases heat; on the other hand the conversion of vitamin D_3 to $preD_3$ is endothermic and therefore absorbs an equal amount of heat. According to Le Chatelier´s principle, a system in equilibrium will counteract if exposed to external change in temperature (Housecroft & Constable 2006) i.e. if the temperature is increased the equilibrium of pre D_3-D_3 will be shifted towards $preD_3$ as the reaction absorbs heat; therefore the ratio of $preD_3$-D_3 at equilibrium depends on temperature. Calculated equilibrium ratios at different temperatures are displayed in Table 2-1 (Hanewald et al. 1961); the values are close to what is observed in solvent where equilibrium is reached with 80 % vitamin D_3 after 2 hours at 80 °C (Takada et al. 1979) while after 2 hours at 90 °C there is 67% in the form of vitamin D_3 (Holick et al. 1979). It takes 13 days at 20 °C to turn 80 % of $preD_3$ into vitamin D_3 if having a pure solution of $preD_3$ while it takes 43 hours at 40 °C and at -20 °C it will take 6 years (Hanewald et al. 1961) and less than 2 % is in the form of vitamin D_3 after 48 hours at 0 °C (Holick et al. 1979).

Table 2-1 Equilibrium ratios of previtaminD_3 and vitamin D_3 in ethanol at different temperatures (Hanewald et al. 1961)

Temperature	% at equilibrium in solvent	
°C	Previtamin D_3	Vitamin D_3
-20	2	98
0	4	96
20	7	93
40	11	89
80	22	78
100	28.5	71.5

However, the reaction rate constants and the equilibrium ratio of vitamin D_3 are higher in skin than what is observed in solvents; this is true both *ex vivo* and *in vivo* (Tian et al. 1994; Tian et al. 1993). The reaction rate is increased more than 10-fold in skin compared to solvent (Holick et al. 1995; Tian et al. 1993). *In vivo* vitamin D_3 is actively transported into circulation pushing the equilibrium $preD_3 \leftrightarrow D_3$ towards vitamin D_3; however because k_2 is much smaller than k_1 this will not affect the rate or equilibrium ratio enough to explain the observed difference between solvent systems and skin (Tian et al. 1993). The triene system in $preD_3$ exist either as cis-cis (cZc) or trans-cis (tZc) (see Figure 2-4), where the tZc conformation is the thermodynamically favoured isomer but only the cZc form can isomerise to vitamin D_3. In solvent 90 % of $preD_3$ is in the tZc form (Keegan et al. 2013) and this explains why it takes 30 hours at 37 °C to isomerise 50 % of $preD_3$ to vitamin D_3 in solvent ($T_{1/2}$ = 30 hrs) while in skin at 37 °C $T_{1/2}$ is only 2.5 hrs (Tian et al. 1993). In skin 7-DHC is mainly located in the phospholipid bilayer of the cell membrane where it has been hypothesised that the hydroxyl group of 7-DHC interacts with the polar head of the phospholipids while the non-polar rings and side-chain of 7-DHC interacts with the hydrophobic tails (see Figure 2-4); when $preD_3$ is produced from 7-DHC in this locked position the cZc conformation is stabilised, as it would require energy to break the hydrophilic interaction, thereby the reaction rate is increased compared to pure solutions (Holick et al. 1995). In phospholipid bilayers formed *in vitro* the conversion of $preD_3$ to vitamin D_3 is 6-12 times faster than in hexane that is consistent with the findings in skin (Yamamoto & Borch 1985). Holick et al. (1995) suggests that the conversion of $preD_3$ to vitamin D_3 disrupts the interaction with the phospholipid bilayer whereby vitamin D_3 is translocated from the cell wall into the extracellular space.

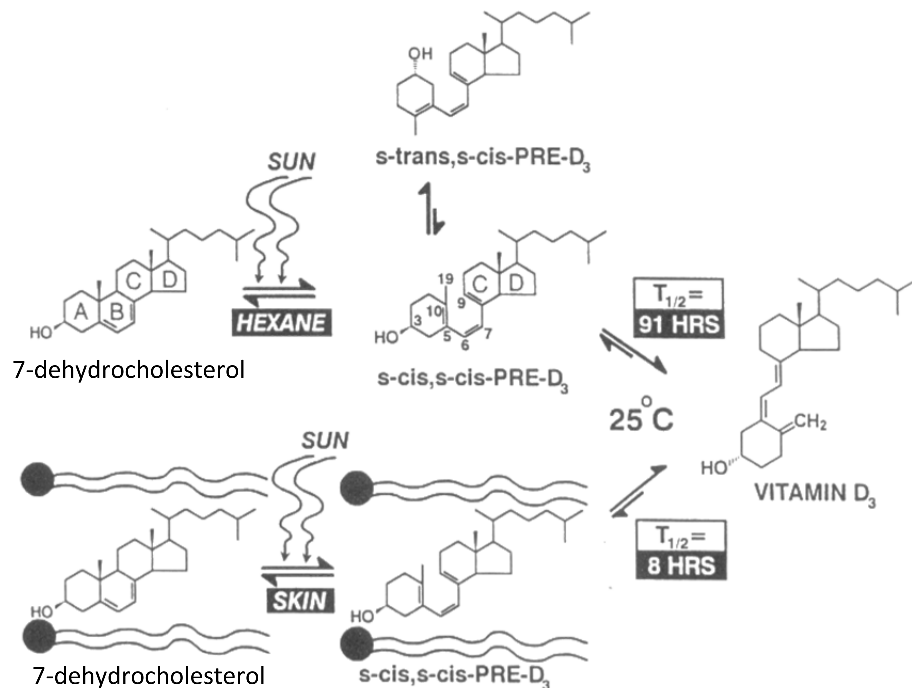


Figure 2-4. Photoproduction of previtamin D3 (PreD3) from 7-dehydrocholesterol and its subsequent isomerisation to vitamin D3 in hexane and in skin. The cis-cis conformation of preD3 is thermodynamically unfavourable in solvent and the major part of preD3 will isomerise to the favoured trans-cis form, however the cis-cis form is stabilised in the phospholipid bilayer in the skin (Figure from Holick et al. (1995)).

Comparing the amount of vitamin D_3 in human skin *in vivo* and *ex vivo* post UVB exposure there is 11 % less *in vivo* 30 minutes after exposure while there is 26 % less after 60 minutes. The most probable explanation is that *in vivo* vitamin D_3 is actively transported into circulation (Holick et al. 1980; Tian et al. 1993).
90 % of preD_3 is transformed into vitamin D_3 within 16 hrs at 40 °C in chicken skin (*ex vivo*) (Tian et al. 1994).The percentage of formation is comparable in skin from humans, frogs, lizards and chickens (Holick et al. 1995; Tian et al. 1993).

2.8 Biological activity of the vitamin D vitamers

The biological activity of vitamin D and its metabolites has historically been determined in animal assays by use of different endpoints e.g. calcification of bones, calcium uptake and total bone ash. Currently serum 25(OH)D_3 level is the generally accepted biomarker for vitamin D status.
In some food databases, including the Danish, a factor of 5 is assumed for the biological activity of 25(OH)D_3 although there is little scientific evidence for this factor and it is based on animal studies (Jakobsen 2007). Based on 7 randomised controlled

trials (RTCs) in humans the average biological activity of $25(OH)D_3$ is calculated to be around 3.2 in a recent review by Quesada-Gomez & Bouillon (2018), the range was between factor 1.04 and 5.5. One RTC that was published at the same time as the review, and therefore not included, found a factor of 1.5 (Jakobsen et al. 2018). The factor increases with increased dose from 1.04 at 5 µg/day, 1.5-5.5 at doses below 25 µg/day and 8 (average of 3 studies) at doses above 50 µg/day (reviewed by Quesada-Gomez & Bouillon (2018); (Jakobsen et al. 2018)); the daily intake of $25(OH)D_3$ from food is assumed to be less than 5 µg/day and therefore a factor of 1 might be the best estimate when calculating total vitamin D in food.
The biological activity of preD has been determined in two studies; one using 140 rats in each group and the other using 26-28 chickens in each group. The studies showed that preD has 34% to 35 % the anti-rachitic activity of vitamin D when fed to the animals in pure solution (Bekemeier & Pfordte 1963; Hanewald et al. 1961). Shaw & Jefferies (1957) reported the activity to be 40 % but states that it is from a small and unreliable trial. While Bolliger (1965) reported that own trials had shown the anti-rachitic activity of $preD_2$ to be 56 % but included no description of the trial. For this reason it is assumed that the biological activity of $preD_3$ is 35 % that of vitamin D_3. The reason for the observed anti-rachitic activity of preD is most likely due to isomerisation of preD to vitamin D inside the host after ingestion (Hanewald et al. 1961; Shaw & Jefferies 1957).

3. Quantification of vitamin D in food

With the discussion of which level of vitamin D is adequate in foods and what the actual intake of vitamin D is, it is necessary to be able to analyse the amount of vitamin D in different foods. LC-MS methods for the analysis of vitamin D in food mostly consist of a saponification step (hot or cold), a liquid-liquid extraction (LLE) step and a solid-phase-extraction (SPE) step. LC-ESI-MS (high performance liquid chromatography electrospray ionisation mass spectrometry) methods typically also include derivatisation with PTAD, use vitamin D_3-d_6 as internal standard and the eluent additives are formic acid alone or in combination with methylamine or ammonium formate alone (Schadt et al. 2012; Lietzow et al. 2012; Lipkie et al. 2013; Burild et al. 2014; Gomes et al. 2015; Barnkob et al. 2019) while the LC-APCI-MS methods either uses vitamin D_3-d_3 or vitamin D_2 as IS (Heudi et al. 2004; Byrdwell et al. 2008; Dimartino 2009; Höller et al. 2010; Trenerry et al. 2011; Bilodeau et al. 2011; Huang et al. 2012; Gilliland et al. 2012; Stevens & Dowell 2012; Strobel et al. 2013; Byrdwell et al. 2013).
Three different analytical methods have been used in this thesis all of which used cold saponification. It has however recently been advised by (Gill et al. 2019) to avoid cold saponification. Therefore the saponification step will be discussed followed by a presentation of the methods used along with the reason why three different methods were necessary. Differences between the methods were observed and investigated and this is the main focus of chapter 3 (**Paper IV**). The chapter is completed by a discussion of the findings presented and future perspectives. The chapter begins with a small introduction to the theory behind ESI-MS/MS to facilitate the understanding of the rest of the chapter.

3.1 Theory behind ESI-MS/MS

3.1.1 ESI

The following description is based on the introductory description of ESI in Konermann et al. (2013). Compounds separated using LC is in liquid form, while MS analyses ions in gas phase. ESI is one method to get analytes into gas form, while at the same time being ionised. The liquid flow from the LC is passed through a heated capillary into a heated chamber where there is an electrical field between the tip of the capillary and a counter electrode located at the entrance to the MS. In positive mode redox reactions

at the tip of the cone will provide an excess of positive charges, mainly in the form of protons. The electrical field will draw out the positively charged liquid into what is known as a Taylor cone and at the tip of the cone a fine mist of droplets is emitted. The droplets will decrease in size as the solvent evaporates, often assisted by a coaxial flow of heated nitrogen gas. As the solvent evaporates the charge is build up inside the droplet until the surface tension is balanced by the Coulombic repulsion, this is known as the Rayleigh limit. At this limits smaller droplets are produced by jet fission and this process goes on until all the solvent is evaporated and the analyte is then in gas phase as a positively charged species due to transfer of charge, e.g. adduct formation with a proton. An illustration of the process described above is shown in Figure 3-1.

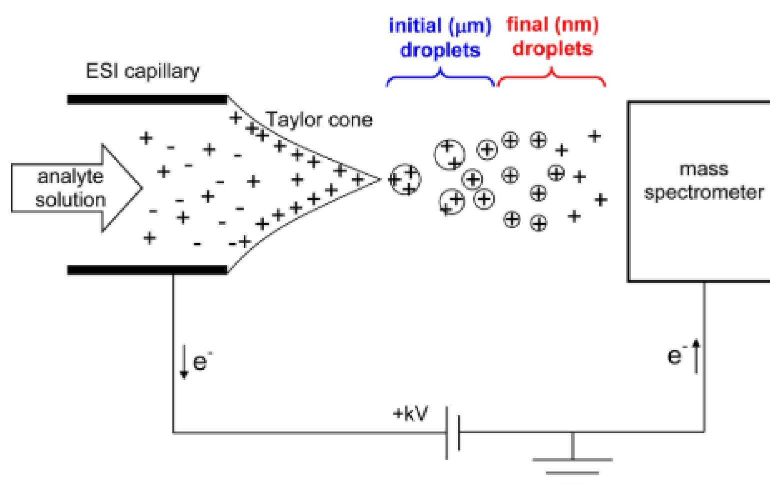


Figure 3-1 Schematic illustration of the production of ions in an ESI source. From Konermann et al. (2013) with permission.

3.1.2 MS/MS

A MS/MS consists of two mass spectrometers with a collision cell in-between. In SRM (selected reaction monitoring) mode the first MS selects the precursor ion of interest according to the mass to charge ratio (*m/z*), the precursor ion is fragmented into product ions in the collision cell by collision with nitrogen gas; one of the precursor ions is selected according to its *m/z* in the second MS before it is detected. In MRM (multiple reaction monitoring) mode multiple product ions from one or more precursor ions are detected. MS/MS is highly selective and when using MRM or SRM only the peaks of interest will be seen.

3.2 Saponification – an essential step in the analysis of vitamin D

It is estimated that fortified foods can contain significant amount of preD_3, up to 8 % of the total vitamin D content has been determined to be in the form of PreD_3 in vitamin D premixes (Gill et al. 2019); and it is estimated that the preD_3 content could be as high as 30 % of the total vitamin D_3 in some cases (Huang et al. 2014). It is however unknown if inherent vitamin D in foods, like egg yolk, also contain preD_3.

It would be preferable to have a method that could quantitate both preD_3 and vitamin D_3 separately, however so far there is no commercially available internal standard for preD_3 (Yang et al. 2017).

As described in Section 2.7 the ratio between preD_3 and vitamin D_3 depends on time and temperature. SIL IS is added under the assumption that it will be converted to preD_3 at same rate as the analyte; however, SIL IS is almost in pure vitamin D_3-form while food samples possibly contain significant amount of preD_3 as described above. For this reason the time and temperature of the saponification step in the extraction of vitamin D is essential, as it takes 53 hours to convert 2 % of a pure solution of vitamin D_3 into preD_3 at 20 °C while it take 3.7 hours at 40 °C (Hanewald et al. 1961) and 2 hours at 80 °C (Gill et al. 2019).

Gill et al. (2019) equilibrated a pure vitamin D_3 solution at 20 °C and then simulated saponification, at different temperatures, of the equilibrated solutions (sample) and a standard solution and determined the vitamin D_3 content in both. For simulated cold saponification (25 °C) the trial ran for 12 days with samples collected for analysis every 24 hours while for simulated hot saponification (60-80 °C) samples were taken out every 15-60 minutes with trial period between 180-720 minutes. The equilibrated solution contained 91.8 % vitamin D_3 and the standard contained 100% vitamin D_3. It was estimated that after 12 hours of simulated cold saponification the ratio between vitamin D_3 in the sample and the standard had changed with 0.8 % points during cold saponification; however only the content of vitamin D_3 is determined and there is around 8 % preD_3 that will not be determined. After hot saponification to equilibrium the ratio is close to 1, and thereby the total vitamin D (sum of vitamin D_3 + preD_3) is determined; Gill et al. (2019) therefore conclude that cold saponification should be avoided. However, remembering that the biological activity of preD_3 is 35 % that of vitamin D_3 (see section 2.7) the active vitamin D content is underestimated by approximately 3 % when using cold saponification while it is overestimated with approximately 5 % when using hot saponification if the initial content of preD_3 is 8 %.

The change in ratio of 0.8 % points at cold saponification shown by Gill et al. (2019) is assumed to be within the analytical error and not necessarily due to an actual change. In general terms cold saponification will underestimate the total vitamin D activity with 35 % of the initial content of preD_3 and hot saponification (to equilibrium) will overestimate it by 65 % of the initial content of preD_3. Cold saponification is therefore the most accurate method of the two.
If using hot saponification using vitamin D_3-d_3 should be avoided as the deuterium is placed at the centre of isomerisation between preD_3-D_3 and therefore give biased results as shown by Huang et al. (2014); Vitamin D_2, vitamin D_3 and vitamin D_3-d_6 have equal isomerisation rates and equilibria (Hanewald et al. 1968; Woollard et al. 2019; Huang et al. 2014).

3.3 Analytical methods used in the project

When I started my project there was already a LC-ESI-MS/MS method for the determination of vitamin D_3 and 25(OH)D_3 in foodstuff (Burild et al. 2014). This method used methylamine and formic acid as eluent additives. However, during the run of the first egg yolk samples the MS/MS broke down beyond repair. Therefore the rest of the egg yolk samples and pig skin samples were extracted by alkaline saponification overnight at room temperature and cleaned up using liquid-liquid extraction (LLE) and solid-phase extraction (SPE) according to Burild et al. (2014) followed by preparative HPLC (as described in Kristensen et al. (2012)) and analysed on HPLC-UV-DAD (HPLC coupled to a UV and photo-diode array detector) according to the method described in Jakobsen et al. (2007). The analysis of the skin samples is described in **Paper I**. As for the quality control in the analysis of the yolk samples an in-house reference (egg yolk) was analysed each day of analysis showing an internal reproducibility CV of 11 % (n=10).The vitamin D_3 level in the in-house reference determined using either LC-MS/MS or UV were the same according to the in-house quality control X-chart.
When I analysed the samples from the pig trial a new MS/MS had been acquired and a new method had been developed where the methylamine/formic acid had been replaced by ammonium formate; this method is described in **Paper II**. However the method was not completely robust and especially for eggs the vitamin D_3 result had to be corrected for recovery; this had never been the case with the previously used

methods and was also quite remarkable as vitamin D_3-d_6 was used as internal standard.

Both LC-MS/MS methods include a step where the vitamers are derivatised with the Cookson-type reagent 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). PTAD selectively reacts with conjugated dienes. Both vitamin D_3, 25(OH)D_3 and 1,25$(OH)_2D_3$ have such a diene system in the same location. PTAD increases the ionisation efficiency of the vitamers and when analysed using SRM both PTAD-25(OH)D_3 and PTAD-vitamin D_3 produces a characteristic 298 *m/z* fragment (see Figure 3-2) that reduces background noise (Aronov et al. 2008) . From now on when the vitamers are mentioned in regards to LC-MS/MS analyses it is therefore implicit that it is the PTAD-vitamers that are used.

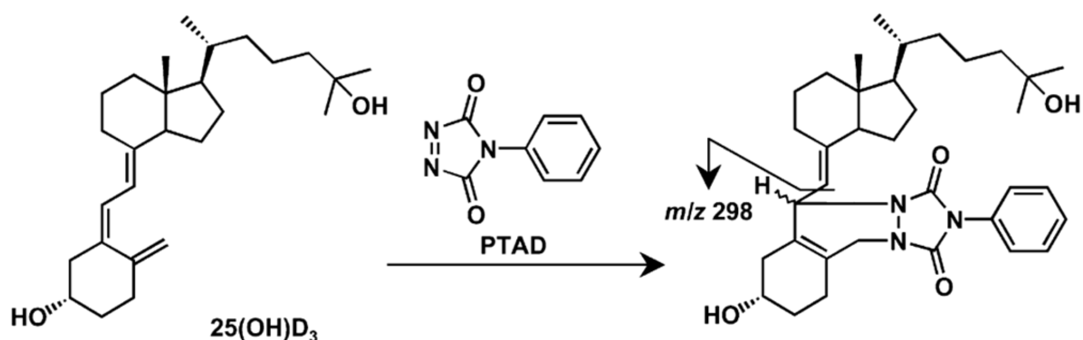


Figure 3-2 Reaction of 25-hydroxyvitamin D_3 with PTAD. The arrow in the structure to the left indicate the major fragment (*m/z* 298) obtained in SRM when using LC-ESI-MS/MS. From Higashi et al. (2010) with permission.

Results from the analyses are presented as mean ± standard deviation (SD) throughout the thesis.

3.3.1 Effect of changing the eluent additive on the quantification of vitamin D_3

The gradient of the ammonium formate method was changed without any luck. The MS/MS was ruled out as a factor by running the methylamine method on the new instrument and obtained similar results as earlier on the old MS/MS; however remnants of methylamine remained in the system and decreased the signal of vitamin K by approximately 50 % when analysed in subsequent runs. The difference between using methylamine/formic acid and ammonium formate as additive was therefore investigated; besides the obvious that the protonated adduct was used as precursor when ammonium formate was added and the methylamine adduct was used when methylamine/formic acid was added. The pH of the 5 mM methylamine and 0.1 %

formic acid measured in water was 3.2 while the pH of 2.5 mM ammonium formate in water was measured to be 6.1. Formic acid has a pKa of 3.75 while ammonium has a pKa of 9.25, ammonium formate will therefore not function as a buffer at pH 6.1 (Konermann 2017). During ESI water is oxidized whereby the pH can be lowered by up to 4 pH units; however the pH is most affected in systems that are unbuffered and around neutral pH (Van Berkel et al. 1997). Adding 0.05 % formic acid in addition to the 2.5 mM ammonium formate in water lowered the pH to 3.2 that was within the buffering capacity. When reference samples were analysed using a combination of ammonium formate and formic acid in the eluent the quantitation of vitamin D_3 were almost similar to the method using methylamine. The method, however, was still not robust. It was judged that MEs were the cause of the problems and that they were affected by the eluent additive. Therefore a new task was to investigate how different eluent additives affected the ionisation of vitamin D_3 and vitamin D_3-d_6.

3.4 Investigating the effect of different eluent additives in vitamin D_3 analysis (Paper IV)

3.4.1 Deuterated SIL IS does not always correct for matrix effects

When using MRM or SRM in MS/MS only the peaks of interest are observed, however interferences not detected can still have an impact on the signal. In the case of ion suppression of the analyte/IS the LOD will be higher (Buhrman et al. 1996) and if the IS and analyte does not co-elute it can affect the accuracy of the method. Compounds with the same retention time, precursor and product ion are seldom the cause of interferences in LC-MS/MS; interferences are rather caused by other co-eluting compounds that influence the ionisation of the analyte (Bonfiglio et al. 1999). It is known that co-eluting matrix compounds may cause ion suppression or ion enhancement although the underlying mechanism of the matrix effect (ME) is not yet fully elucidated (Matuszewski et al. 2003). It is generally assumed that SIL IS will remove any relative ME as it is presumed that it co-elute and behave physically and chemically like the analyte (Matuszewski 2006; Matuszewski et al. 2003; Bonfiglio et al. 1999; Wu et al. 2013). However, deuterated (^{2}H) SIL ISs are a little less lipophilic than the corresponding analyte and therefore elutes slightly before the analyte. This difference in retention time (RT) can result in a different degree of ion suppression of the two analogues due to ME, affecting the area ratio and thereby the determined content (Wang et al. 2007; Wieling 2006).

3.4.2 Determination of matrix effects

Reviews of methods for evaluating ME have been published elsewhere (Furey et al. 2013; Trufelli et al. 2011). Using recovery based methods for detecting interferences does not give information on the chromatographic profile of the interference or of late eluting interferences that if not eluted can affect subsequent runs (Bonfiglio et al. 1999). A full scan of a sample can sometimes give information about interfering compounds, however interferences cannot necessarily be detected by MS (Bonfiglio et al. 1999). The only method that also includes the chromatographic profile is the post-column infusion method which was first described by Bonfiglio et al. (1999). The post-column infusion method requires a matrix blank; however, this is hard to come by when analysing nutrients in food. Instead I assumed that disregarding the chromatographic separation, ME will affect the ionisation of vitamin D_3-d_6 and vitamin D_3 in the same manner; so I came up with the idea of using continuous post-column infusion of vitamin D_3-d_6 to visualise ion suppression when no blank matrix is available.

3.4.3 Continuous post-column infusion of vitamin D_3-d_6 to visualise ion suppression

Continuous post-column infusion of PTAD-vitamin D_3-d_6 was used to visualise the ion suppression of different matrices and how it was affected by eluent additives.
The setup and sample preparation is described in **Paper IV**. Figure 3-3 shows the post-column ion suppression chromatograms obtain from running sample extracts of salmon, cheese, egg yolk and liver. The blue chromatograms were obtained using an eluent containing 2.5 mM ammonium formate alone and the pink is from the combination of 2.5 mM ammonium formate and 0.05 % formic acid. The vertical black line shows the retention time of vitamin D_3; vitamin D_3-d_6 elutes 0.019 min before vitamin D_3. From Figure 3-3 it is seen that the different matrices gave different ion suppression patterns and a lot of suppression was observed between 5-7 min. Adding 0.05 % formic acid to the eluent decreased the variation of the ion suppression (pink) and thereby decreases the difference in the ionisation efficiency of the vitamin D_3 analogous. Somehow the liver matrix was not affected by the addition of formic acid to the eluent, making the two chromatograms almost the exact same; and the ion suppression was almost completely stable during the elution of the vitamin D_3 analogous.

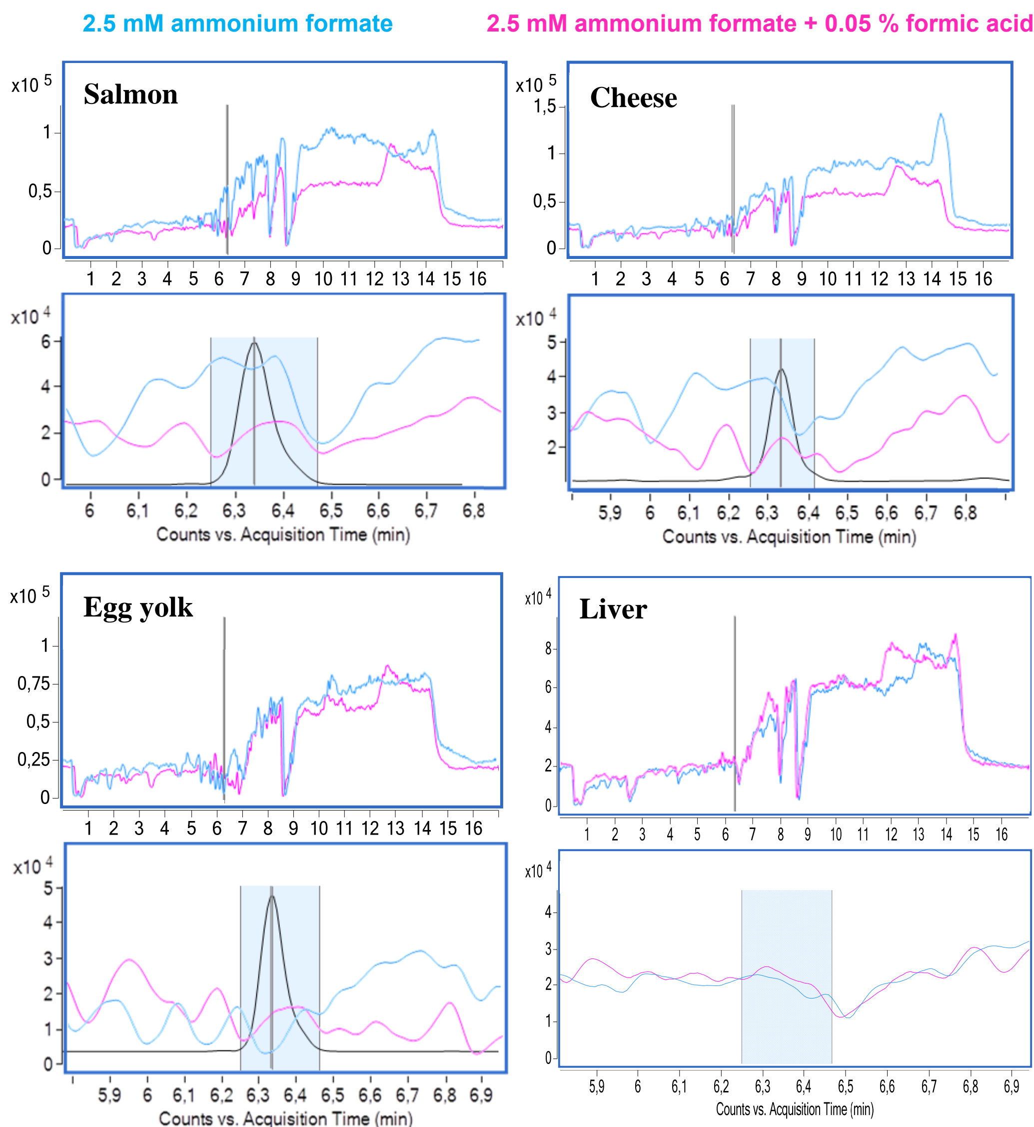


Figure 3-3 Post column infusion of *d6*-vitamin D_3 (566.3 *m/z* → 298.1 *m/z*) obtained during running of extracts of salmon, cheese, egg yolk and pork liver. The full chromatograms and zoom-in on the retention window of vitamin D_3 is shown. The vertical line in the full chromatogram shows the retention time of vitamin D_3 while the zoom-in is overlaid with the vitamin D_3 peak (560.3 *m/z* → 298.1 *m/z*). The blue suppression pattern was obtained using an eluent with 2.5 mM ammonium formate. The pink was obtained using an eluent with 2.5 mM ammonium formate and 0.05 % formic acid.

From Figure 3-4 it is seen that the suppression patterns of SRM 1546a meat homogenate, pork loin from 2006, pork loin from 2016 and beef are comparable when 2.5 mM ammonium formate was used alone. At the retention time of the vitamin D_3 analogous the ion suppression was relatively stable. When adding 0.05 % formic acid to the eluent containing 2.5 mM ammonium formate the suppression patterns became nearly identical.

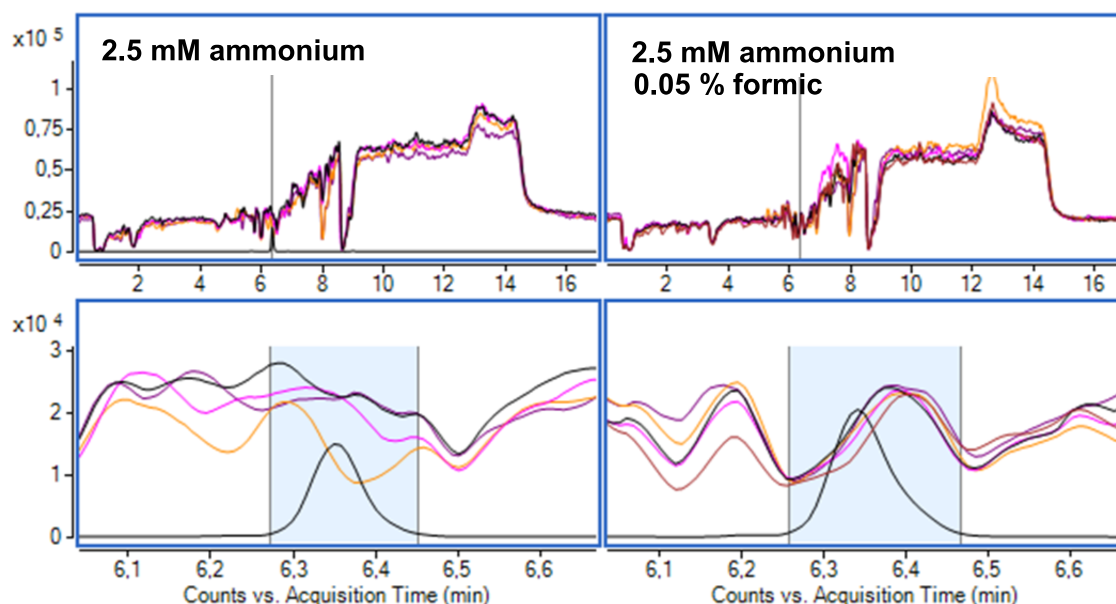


Figure 3-4 Post-column infusion chromatograms of vitamin D_3-d_6 (566.3 *m/z* → 298.1 *m/z*) during injection of extracts of Nist® SRM® 1546a meat homogenate , pork loin from 2006, pork loin from 2016, beef and pork fat. The vertical line in the upper full chromatograms shows the retention time of vitamin D_3 while the lower chromatograms (zoomed in) are overlaid with the vitamin D_3 peak (560.3 *m/z* → 298.1 *m/z*). The run of pork fat failed in the run with 2.5 mM ammonium formate and was therefore omitted.

It was not possible to explain the big difference obseved in recovery between the eluent with ammonium formate and ammonium formate/formic acid from the post-column infusion chromatograms. It was however visualised that ion suppression due to ME was quite pronounced for most matrices especially around the retention time of the vitamin D_3 analogous. When comparing the infusion chromatograms of the different food samples it was clear that a method for determining vitamin D in food is not per se universal and ME have to be assessed in all types of food samples one might want to analyse. However, the ion suppression observed in pork meat, pork fat and beef meat was comparable therefore it could be possible to make a full validation on only one of those matrices.

Figure 3-5 displays the ion suppression of the egg yolk extract and for SRM 1546a and pork loin (2006) during a run with methylamine/formic acid in the eluent; the ion suppression patterns looks simpler (compared to Figure 3-3 and Figure 3-4) with more defined suppression peaks. However, there are still a lot of suppressions close to the retention time of the vitamin D_3 analogues but they elute in a relatively steady area in between two suppression regions as seen from the zoom-ins (in Figure 3-5) and this explains why the recovery of this method is acceptable.

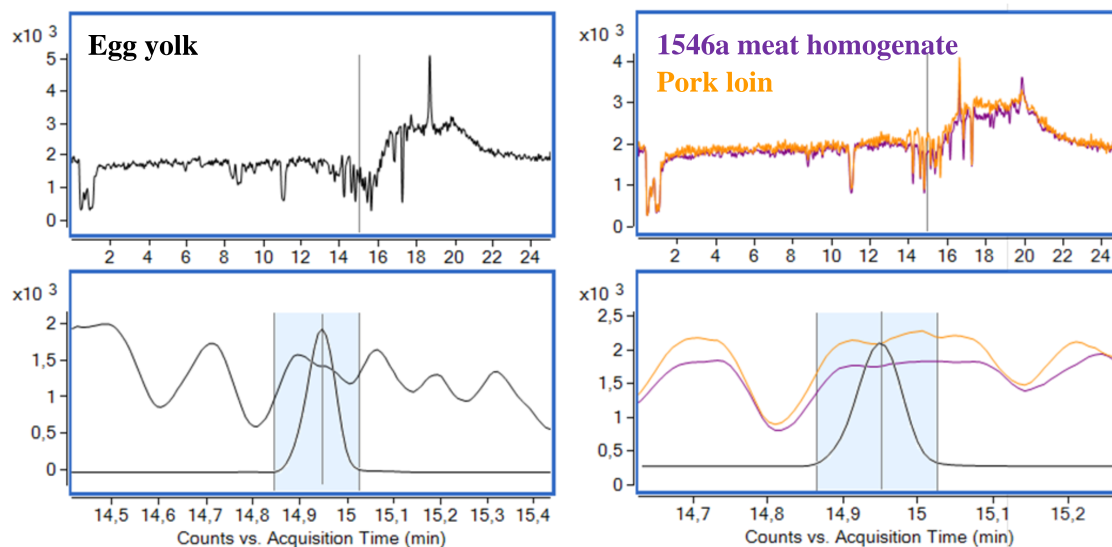


Figure 3-5 Post-column infusion chromatograms of the methylamine adduct of vitamin D_3-d_6 ($[M+CH_3NH_3]^+$ 597.4 *m/z* → 298.1 *m/z*) obtained during run of extracts of egg yolk, 1546a meat homogenate and pork loin. The eluent contained 5 mM methylamine and 0.1 % formic acid and a segmented linear gradient with 60% to 100 % MeOH was used.

3.4.4 Effect of eluent additives on adduct formation

It was also looked into how ammonium formate and formic acid affected the formation of adducts. The proton, sodium and ammonium adducts were selected for product ion scan. Only the protonated adduct produced a product ion with significant abundance, the other adducts remained intact during collision. When no additives were added to the eluent the most abundant ion was the sodium adduct (see Table 3-1). Adding ammonium formate alone increased the abundance of the protonated adduct significantly and its product ion was increased more than 40 times; the ammonium adducts were reduced while the sodium adduct was not affected. Adding formic acid alone decreased the abundance of all the ions by at least 50 %. Adding both ammonium formate and formic acid to the eluent maintained the effect of formic acid on the sodium and ammonium adducts while the effect of ammonium formate on the protonated adducts and its product ion was significantly reduced.

Table 3-1 The abundance of the proton, sodium and ammonium adduct of vitamin D_3 at different combinations of ammonium formate and formic acid in the eluent. Determined using product ion scan from 150 *m/z* to 600 *m/z,* collision energy 10. Only the protonated ion gave rise to a product ions with high abundance, the other adducts stayed intact during collision.

Eluent additive (mM)		Abundance (x 10^4)				
ammonium formate	**Formic acid**	**[M+H]+→298.1**	**$[M+H]^+$**	**$[M+Na]^+$**	**$[M+NH4]^+$**	**$[M+H+NH4]^{2+}$**
0 mM	0 %	2.0	2.4	14	2.3	3.3
2,5 mM	0 %	88	11	17	0.15	0.37
2,5 mM	0,05 %	9.1	2.8	5.7	1.6	1.1
0 mM	0,05 %	1.0	1.1	5.7	1.4	2.1

That formic acid can be used to reduce the abundance of sodium adducts has also been mentioned in a review (Kostiainen & Kauppila 2009). The ionisation of 25(OH)D_3 is also lower when 0.1 % formic acid is used as the only eluent additive compared to using it in combination with 10 mM ammonium formate (Ding et al. 2010). The effect of eluent additives is compound specific; an additive can enhance the ionisation of one compound while enhancing another (Mallet et al. 2004); ammonium formate enhanced the ionisation of vitamin D_3 but it has been shown to decrease the ionisation of 8 different pharmaceutical compounds (Mallet et al. 2004).

3.5 Discussion of results and future perspectives for improvement of the analytical method

From the results it was clear that due to its earlier retention time deuterated SIL IS cannot fully eliminate ME when analysing vitamin D_3 in most food matrices. Adding ammonium formate to the eluent greatly increased the formation of the characteristic product ion with 298.1 m/z (see section 3.4.4) and thereby the sensitivity was increased; however, the robustness and accuracy of the method when using it alone was not acceptable and it was very prone to ion suppression (see section 3.4.3). Using a combination of 5 mM methylamine/0.1 % formic acid as eluent additive has been shown to provide an acceptable accuracy, but methylamine remains in the HPLC system and affects the analysis of vitamin K in subsequent runs and it is therefore preferable to avoid the use of it. The pH of the ammonium formate eluent was adjusted to the same pH as the methylamine/formic acid eluent by adding 0.05 % formic acid; the accuracy was improved, but not the robustness; the variation of the ion suppression within the retention window of the analyte was decreased but no near to the same degree as observed when using methylamine/formic acid. The accuracy and the

robustness could most likely be increased by using a ^{13}C labelled SIL IS instead, as it has the exact same retention time as the corresponding analyte and thereby the ratio between SIL IS and analyte will not be affected by ME.

The sensitivity of the method could most likely be increased by optimising the composition of the eluent additives. The effect of using methylamine, ammonium formate and formic acid as eluent additives in the analysis of 25(OH)D_3 has been studied by others (Higashi et al. 2008; Ding et al. 2010) Addition of methylamine (5 mM) to the mobile phase also provide the methylamine adduct $[M+CH3\text{-}NH3]^+$ of 25(OH)D_3 as the base peak and it significantly reduces the occurrence of other adduct ions (Higashi et al. 2008). Ding et al. (2010) investigated the abundance of 25(OH)D_3 using four combinations of eluent additives and found that the abundance was highest when using 5 mM methylamine/0.1 % formic acid > 5 mM methylamine/10 mM ammonium formate/ 0.1% formic acid > 10 mM ammonium formate/ 0.1% formic acid > 0.1% formic acid. However, comparing 5 mM of one additive to 10 mM of another is not advisable as according to the results of(Mallet et al. (2004) the effect of eluent additives also depends on concentration of the additive e.g. at low concentration an additive can enhance ionisation of an analyte, while at higher concentrations the ionisation can be suppressed; and in general ionisation efficiency decreases with increasing concentration of eluent additive. When analysing the vitamin D content in food both vitamin D_3 and 25(OH)D_3 should be included; however, the ionisation efficiency of the two might be affected differently by eluent additives. For this reason a systematic investigation using different additives, at different concentrations and combinations, where the ionisation efficiency of both vitamin D_3 and 25(OH)D_3 is taken into consideration, is suggested.

Furthermore it would be preferable to have a method that could quantitate both preD_3 and vitamin D_3 separately, however so far there is no commercially available internal standard for preD_3 (Yang et al. 2017). However PTAD-preD_3 does produce other products ions than PTAD-vitamin D_3 (Mahmoodani et al. 2017; Yang et al. 2017) so if a chromatographic procedure where preD_3 and vitamin D_3 co-elute (as in Gilliland et al. (2012)) is used then it might be possible to quantitate preD_3 with ^{13}C labelled vitamin D_3 as IS using LC-ESI-MS/MS.

4. Artificial UVB for vitamin D production

This PhD project was part of work package 5 (WP5) of the EU-project ODIN: Food-based solutions for optimal vitamin D nutrition and health through the life cycle. The main objective of WP5 was to explore technological solutions in the food sector which should lead to food products with higher vitamin D content. The purpose was to close the gap between the current intakes of vitamin D in the European population and dietary recommendations and thereby help prevent vitamin D deficiency. This PhD project contributed with investigations of the potential for producing vitamin D enhanced eggs and pork by exposing hens and pigs to artificial UVB. In this chapter the choice of UVB source will be discussed and the study regarding the feasibility of UVB LED for vitamin D production (**Paper I**) will be presented.

Aikaterini Argyraki from DTU Fotonik and I equally contributed to the experimental design of **Paper I**. Aikaterini Argyraki was responsible for the LED UVB measurements and assembling of the lamps and I was responsible for the sample preparation and vitamin D analysis. For this reason there are overlapping content with the thesis of Aikaterini Argyraki (Argyraki 2017).

4.1 Choice of UVB source

In the description of WP5 it was stated that UV light-emitting diodes (LED) with a spectrum equal to sunlight should be used as UVB source for the UVB exposure of hens and pigs. Exposure to the whole light spectrum of the sun have other health benefits as well, whereas narrow-band UVB only is good for vitamin D production (Holick & Hossein-Nezhad 2017). The cost of such a lamp was however too high and it was therefore decided to use the optimal wavelength for vitamin D production instead. The feasibility of using UVB LED for the production of vitamin D was therefore first investigated *ex vivo* in mini-pig skin and UVB LED was used for the UVB exposure of hens; however the effectiveness of UVB LED was too low and the cost too high at the time where the pig experiment was run. It was therefore decided to use UVB emitting fluorescent tubes for the pig trial.
UVB LED benefit from low energy consumption, long lifetimes and minimum heating of surroundings (Souza et al. 2015); however up-front costs of installing UVB-LED lighting

systems are currently high but low cost UVB LEDs with high performance are under development (Khan et al. 2019).

4.2 The optimal wavelength for vitamin D production in skin (Paper I)

The optimal wavelength for vitamin D production has previously been determined by exposing rachitic chickens and rats, 7-DHC solutions, ergosterol solutions (vitamin D_2 precursor), human skin, and human skin models to UVB light of different wavelength obtain with monochromators (see Table 4-1).

When measuring the irradiance of a narrow spectrum UVB source and plotting it against the wavelength a peak with Gaussian shape appear; the wavelength corresponding to the top of the peak is the central wavelength and the full width at half maximum (FWHM) is used as a measure of the broadness. It was thought that UVB LED would have a narrower spectrum than other UVB sources previously used to determine the optimal wavelength for vitamin D production in skin and it therefore would be possible to make a more precise determination. The FWHM of the previously used UVB sources was 3 nm or 5 nm (MacLaughlin et al. 1982).

Table 4-1 The optimal wavelength for vitamin D production determined in different sample types as published by others. Information regarding the limit for no production of vitamin D and the full width at half maximum (FWHM**)** of the used UVB source is also included for each reference. From **Paper I** with permission.

Sample type	Optimum (nm)	No production (nm)	FWHM (nm)	Reference
Rachitic chickens	296.7	313	no info	(Maughan 1928)
Rachitic rats	296.7	313	no info	(Bunker & Harris 1937)
Rachitic rats	280.4-302.4	no info	no info	(Knudson & Benford 1938)
Ergosterol	295	340	no info	(Kobayashi & Yasumura 1973)
Rat skin	303	340	no info	(Takada et al. 1979)
7-dehydrocholesterol	295	no info	no info	(Kobayashi et al. 1976)
Human skin	297	>320	3 or 5	(MacLaughlin et al. 1982)
In vitro human skin models	302	315	5	(Lehmann et al. 2001)
In vitro human skin models	302	-	5	(Lehmann et al. 2007)
7-dehydrocholesterol	295	315	1.7	(Olds et al. 2010)

12 UVB LEDs with wavelengths between 280 nm and 340 nm (see Table 4-2) were purchased. The spectrum of each UVB LED was characterised at DTU Fotonik as described in **Paper I**. The broadness of the spectrums were however much higher than

what had been expected as the FWHM were between 9-16 nm, as seen from Table 4-2.

Table 4-2 Central wavelength, full width at half maximum (FWHM) and range of the UVB LEDs used to determine the optimal wavelength for vitamin D production in pig skin. From **Paper I** with permission.

Central wavelength (nm)	FWHM (nm)	Range (nm)
281	11	266-296
285	11	270-300
292	10	280-304
296	9	284-308
300	10	288-312
306	11	291-321
310	10	298-322
313	10	301-325
318	10	306-330
330	16	309-351
336	12	321-351
338	11	323-353

MacLaughlin et al. (1982) constructed a preD_3 action spectrum; however, the experimental background for it is not well described and its validity is still debated (Van Dijk et al. 2016). An action spectrum is made by using different doses at different wavelengths and from that deducing which dose is required at each wavelength to obtain a specified level of preD_3, and then plotting the reciprocal dose against the wavelength (Norval et al. 2010). Instead of making an action spectrum where the amount of preD_3 is pre-set, we instead used a constant dose of UVB (total irradiation (W/m^2) x exposure time (s) = dose (J/m^2)) and measured the production of vitamin D_3 at different wavelengths in order to examine the relationship between wavelength and vitamin D production.

Skin from the back of a mini-pig (from a previous trial, kept in the freezer) was de-haired and the subcutaneous fat was removed before it was cut into pieces of 1 cm x 1 cm. For each wavelength two different doses, 300 J/m^2 and 7000 J/m^2, were used. One piece at the time was exposed, as can be seen from the experimental setup displayed in Figure 4-1. Double determination was used.

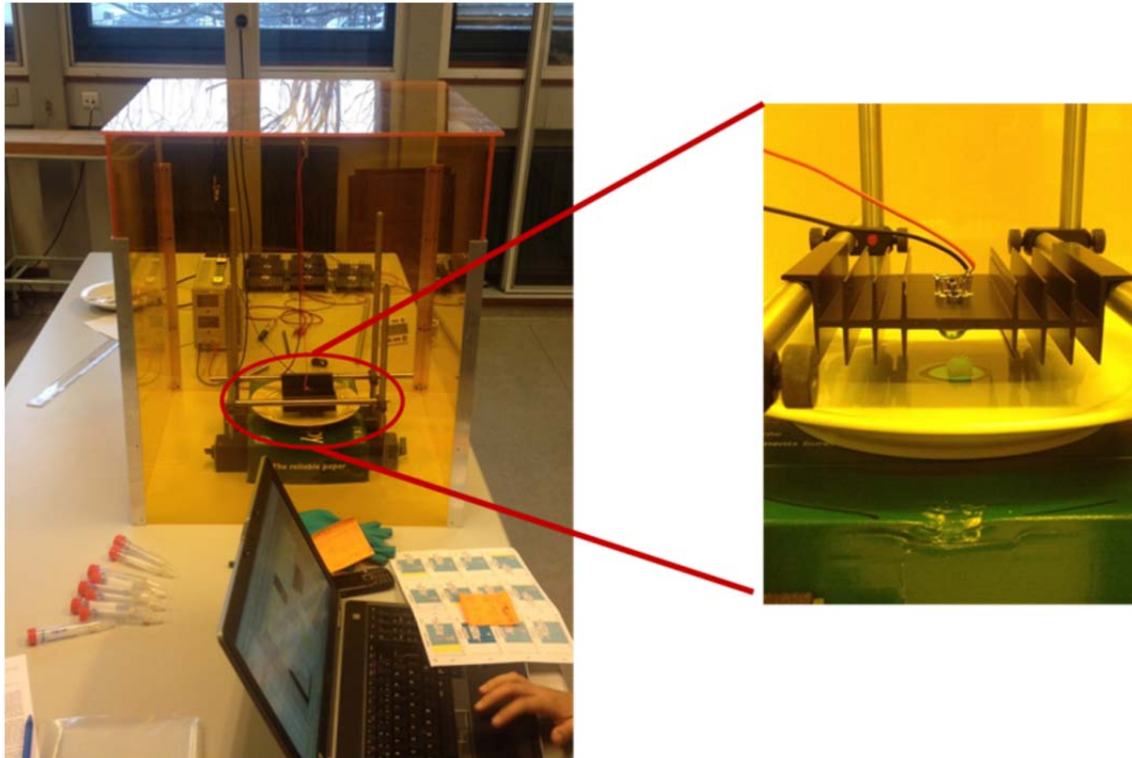


Figure 4-1 Experimental setup for the exposure of pig skin to UVB at different wavelengths. From **Paper I** with permission.

It was planned to analyse the skin samples using LC-MS/MS and also include L_3 and T_3 into the analysis because the ratio between preD_3, L_3 and T_3 was expected to differ between wavelengths (MacLaughlin et al. 1982). As there were no internal standard to quantify preD_3, the skin samples were post exposure put into bags, flushed with nitrogen and sealed before being placed at 37 °C for 24 hours to convert approx. 90-95% of preD_3 to vitamin D_3 (see section 2.7) and was thereafter kept at -80 °C until analysis. Unexpectedly our MS/MS broke down in the middle of the method development for including L_3 and T_3, and it was therefore necessary to quantitate the samples using HPLC-UV-DAD instead, and there unfortunately was not enough time to develop a method that included L_3 and T_3.

The determined vitamin D_3 content in the skin is plotted against the wavelength in Figure 4-2; it can be seen that the maximum production of vitamin D_3 were at 296 nm regardless of the dose.

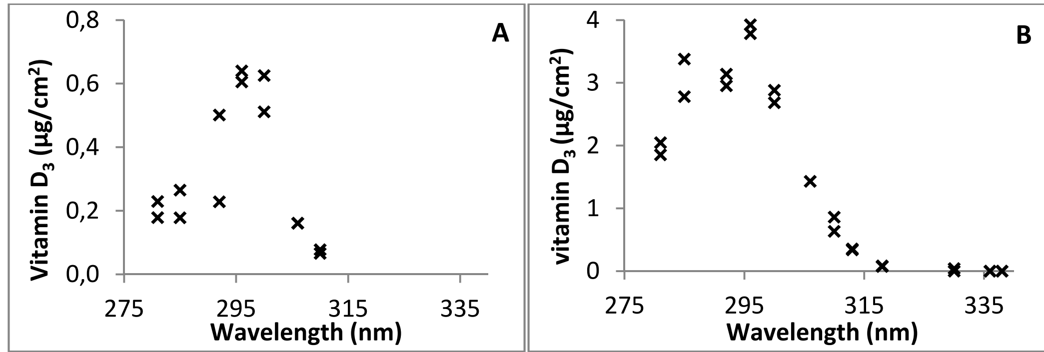


Figure 4-2 Production of vitamin D_3 at different wavelengths determined using a dose of (A) 300 J/m^2 and (B) 7000 J/m^2. From **Paper I** with permission.

A maximum production of vitamin D_3 at 296 nm is in accordance with what is reported to be the optimum wavelength for curing rickets in chickens and rats, and optimum production in solutions of ergosterol (vitamin D_2 precursor) and 7-DHC and human skin; whereas it is a bit lower that what has been reported for rat skin and in vitro skin models (see in Table 4-1). Minimal production of vitamin D was found above 313 nm and no production at wavelengths ≥ 336 nm; this is also in accordance with previously published results as seen in Table 4-1.

4.3 Effect of dose (Paper I)

The effect of dose was determined at the optimum wavelength, 296 nm, using six different doses in the interval 200-20,000 J/m^2. The measured vitamin D_3 as a function of dose first increased linearly, up to approximately 2000 J/m^2, before it started to plateau and the relationship was best described by a logarithmic curve with a correlation coefficient (R^2) of 0.86, as shown in Figure 4-3. The curve in Figure 4-3 resembles a curve presented in MacLaughlin et al. (1982) where human skin (*ex vivo*) had been exposed to UVB at 295 nm with doses up to 30,000 J/m^2; the curve in MacLaughlin et al. (1982) seems to be linear up to a dose of 3000 J/m^2.

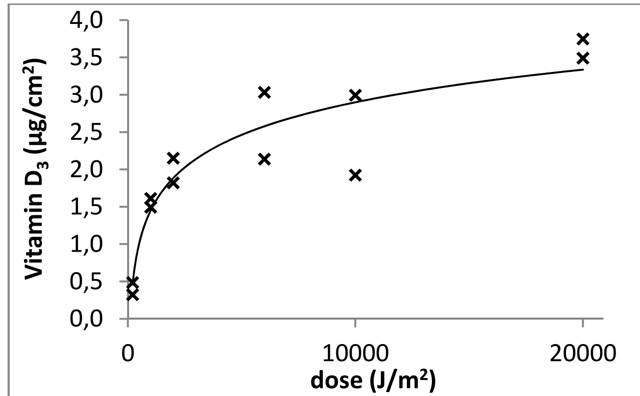


Figure 4-3 The vitamin D_3 content measured in pig skin after exposure to different doses of UVB at 296 nm. From **Paper I** with permission.

4.4 Influence of exposure time and total irradiance (Paper I)

There is an inverse relationship between total irradiance and exposure time at a constant dose according to the equation:

total irradiance (W/m^2) · exposure time (s) = dose (J/m^2).

In order to investigate if the exposure time and total irradiance influenced the production of vitamin D_3, skin samples was exposed to four different irradiances (0.1-43 W/m^2) at three different wavelengths, at an almost constant dose (295-302 J/m^2). The measured vitamin D_3 in the skin samples is shown in Table 4-3. No effect of changing the irradiance was found and thereby neither the exposure time as they are inversely related. It was shown that the dose response relationship was independent of the total irradiance and exposure time. The same conclusion has been reached in a trial where humans were exposed to equal doses of UVB using different exposure times (Bogh et al. 2011).

Table 4-3 Vitamin D_3 in pig skin after exposure to 295-302 J/m^2 at three different wavelengths. For each wavelength four irradiances between 0.1-43 W/m^2 were used. Each exposure was duplicated. P-values were calculated using one-way ANOVA testing for no difference between total irradiances for each wavelength. From **Paper I** with permission.

Wavelength (nm)	Irradiance (W/m2)	Vitamin D_3 (µg /cm^2)		P-value
292	0.1	0.57	0.41	0.62
	3.0	0.46	0.36	
	27.2	0.46	0.45	
	43.0	0.53	0.57	
296	0.1	0.31	0.83	0.96
	3.0	0.43	0.63	
	8.4	0.63	0.61	
	14.8	0.53	0.58	
300	0.1	0.47	0.58	0.06
	2.4	0.71	0.77	
	11.4	0.56	0.43	
	26.8	0.57	0.52	

5. Vitamin D enhanced foods using artificial UVB

The results of the investigations of the potential for producing vitamin D enhanced eggs and pork by exposing hens and pigs (**Paper II**) to artificial UVB is presented in this chapter. Results from direct exposure of egg yolk are also presented. At the end of the chapter the results will be discussed and the results from the hen trial will be compared to published results regarding enhancing eggs through feed (**Paper III**).

Aikaterini Argyraki from DTU Fotonik and I equally contributed to the experimental design of the hen study. Aikaterini Argyraki was responsible for the LED UVB measurements and assembling of the lamps and I was responsible for the sample preparation and vitamin D analysis. For this reason there are overlapping content with the thesis of Aikaterini Argyraki (Argyraki 2017).

5.1 Vitamin D enhanced eggs

As discussed in **Paper III**, it has been shown possible to produce vitamin D enhanced eggs by exposing hens legs to UVB (Kühn et al. 2015; Schutkowski et al. 2013) but not from above (Lietzow et al. 2012) when the hens are supplemented with vitamin D. However, in an industrial setting it is not practical to have lamps directed at the feet. The purpose of the hen trial was therefore to proof that it is possible to produce vitamin D enhanced eggs by exposing hens to UVB from above.

5.1.1 UVB irradiation of hens from above

The original plan was to run two experiments each for the duration of one month and have a weekly collection of eggs. However, due to unforeseen problems the experiment was eventually divided into three parts: two trials with each 7 days of UVB exposure (trial 1 and 2) and one trial with 28 days of exposure (trial 3) as described below.

5.1.1.1 Animals and test environment

40 Lohmann-LSL layers, 28 weeks of age, from a herd of 62.000 hens in enriched cages were supplied by Hedegaard (Denmark). However, all 40 hens were only included in trial 1 while 10 hens were used in trial 2 and 4 hens were used in trial 3.

The hens were placed in the same type of cages as Hedegaard use in the production: Type Euro SO 10 (Hellmann Poultry, Germany), see Figure 5-1. Each cage could house up to 10 hens. The cages were 120.5 cm in length and 62.5 cm in depth. With 10 hens in a cage there were 0.0753 m^2 available per bird. In each cage there was a nesting area (with a curtain in front) and on top of it a scratching area. The trough ran along the complete length of the cage. The cages were linked two and two together, back to back, and shared water supply/drinking nipples. UV opaque material was placed between the cages in order to avoid UV cross contamination between cages.

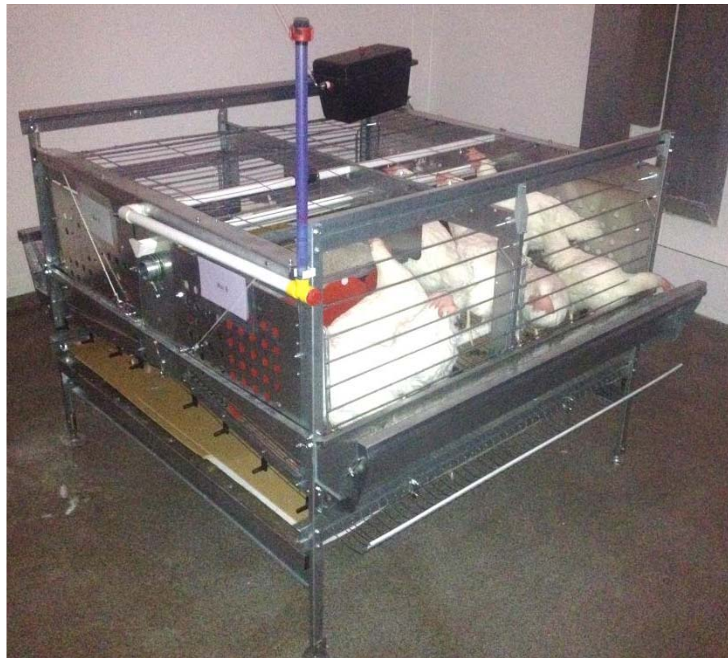


Figure 5-1 Picture of one of the enriched cage units that consisted of two cages back to back. The water nibbles were placed in the middle at the top of the cage (white pipeline) and shared between the two cages. The feeding through were located at lower front of each cage. In the left side of the cages were the nesting area (covered by red plastic curtains) and on top of the nesting area were a sand bath.

The hens were fed a standard feed, which contained the maximum allowed level in the EU, 75 µg/kg vitamin D_3 (Hedegaard Agro, Denmark). Feed and water were offered ad libitum. The feed intake was determined on weekly basis.

The general illumination was on from 01.30-16.30 each day, as in the production facilities. This ensured that the hens laid their eggs in the morning; the hens' laid 1 egg per day per hen. At Hedegaard the light was dimmed to 6 lux. The lighting in the test

facilities came from the same type of fluorescent tubes as used at Hedegaard. There was no dimmer in the existing lighting system, so the light was dimmed to 6 lux by use of white paperboard; this did not change the spectral distribution of the lamps. The illuminance was measured at eye level of the hens by use of a handheld spectrometer UPRtek (MK350, Zhunan, Taiwan).

The acclimatization period was 2 weeks.

Ethical approval was given by The Danish Animal Experiments Inspectorate. The authorization number given was: 2014-15-0201-00254 C1. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use

5.1.1.2 Description of UVB lamps used in the hen trials

The original plan was to have three groups of hens exposed to UVB, however due to high cost it was only possible to purchase UVB-LED enough to install on two cages; an UVB emitting fluorescent tube was therefore installed on the last cage.

UVB LED

Sunlight that reach the surface of the earth does not contain wavelengths below 290 nm (MacLaughlin et al. 1982); for this reason it was decided to avoid wavelengths below 290 nm when exposing live animals to UVB. It was therefore not possible to use UVB LED with the optimal wavelength for the exposure of the hens as the range of the LED with a central wavelength of 296 nm had a range below 290 nm (see Table 4-2). For the exposure of hens 16 UVB LED with an average central wavelength of 306.5 nm and a FWHM of 15 nm were used instead; Figure 5-2 shows the measured full spectrum of each of the diodes from which it can be seen that the amount of wavelengths below 290 nm is negligible. The average range was 287-326 nm. The measurements were performed as described in **Paper I.**

The 16 LEDs were used to construct two assemblies consisting of 8 LEDs each. The two assemblies had a mean total irradiance of 0.76 W/m^2 and 1.06 W/m^2 measured at a distance of 25 cm. However, this difference should not have an impact according to the findings described in section 4.4.

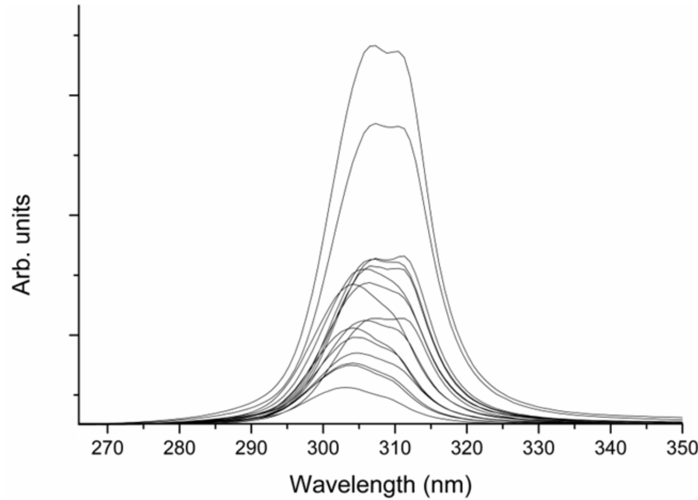


Figure 5-2 Individually measured spectrum of the 16 UVB LEDs with a central wavelength of 306.5 nm, used in the hen trial

UVB emitting fluorescent tube

The UVB emitting fluorescent tube was intended for treatment of psoriasis. The spectral distribution of the UV lamp is shown in Figure 5-3 as measured in the laboratory, at DTU Fotonik, by an irradiance External Optical probe (EOP-146, Instrument systems) and a monochromator (bandpass: 1nm, scan step: 1nm, detector: Photomultiplier). The spectrometer was a SPECTRO 320 (D) Release 5 (Instrument systems). The analysis range was 250-830 nm.The total irradiance in the UVB region (marked with yellow in Figure 5-3) was 2.6 W/m^2 at a distance of 25 cm. In contrast to the UVB LED this tube also emitted visible wavelengths.

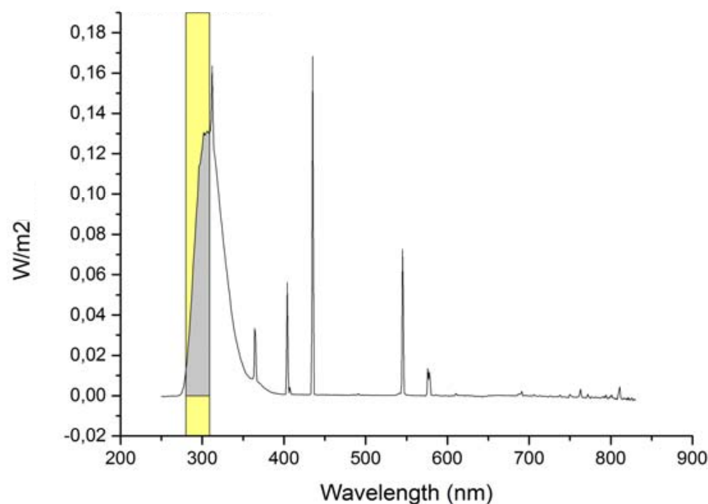


Figure 5-3 Spectral distribution of irradiation delivered by the UVB emitting fluorescent tube used in the hen trial.

Installation of the UVB sources on the cages

The UVB emitting fluorescent tube was placed at the top front of the cage (see Figure 5-4); The fluorescent tube emitted UVB in all directions, so in order to avoid unwanted UVB to reach the other cages UV opaque material was placed on top of the tube as can be seen in Figure 5-4. The two UVB LED assemblies were installed at the top front of the cages, as shown in Figure 5-4. The average distance between the hens and the UVB sources was 25 cm based on observations of the hens' movement pattern in the cages.

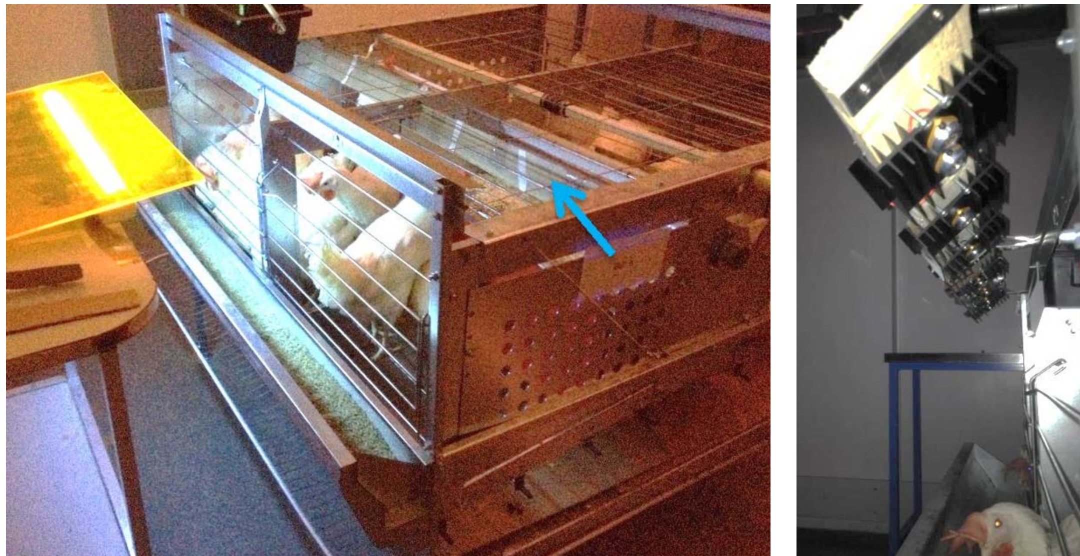


Figure 5-4. To the **left** the placement of UVB emitting florescent tube is seen; the yellow plastic placed on top of the tube is UVB opaque material to avoid UVB from being spread to the entire room. A strip of paperboard (indicated by a blue arrow) was attached at the top of the cage to shield the nibble system (shared by two cages) from UVB. The picture to **right** shows the installation on the cage of one of the two UVB LED assemblies.

5.1.1.3 Sample preparation and analysis

Collected eggs were kept at 5 °C before the yolks were separated from the whites, homogenised, flushed with nitrogen and stored at -20 °C until analysis. All yolks were prepared individually and analysed for vitamin D_3 and 25(OH)D_3. Two different methods (LC-MS/MS and HPLC-UV-DAD) were used for analysis; they are described in Section 3.3. As quality control an in-house reference (egg yolk) was analysed each day of analysis showing an internal reproducibility of 11 % (n=10). The vitamin D_3 level in the in-house reference determined using either MS/MS or UV were the same according to the in-house quality control X-chart.

5.1.1.4 Vitamin D content in eggs at the start of the trial

Sample collection

80 eggs from the production at Hedegaard were delivered along with the hens at the start of the trial, of those 13 were randomly selected for analysis. The plan was to collet eggs after the 2 week acclimatisation period, before starting the UVB exposure, to compare the content with those from the production; these eggs were however lost due to a mistake in labelling of the collected eggs.

Results

The average content of vitamin D_3 in whole eggs, taken from the production site at the day of delivery of the hens, was 1.4 ± 0.4 µg/100 g (n=13, range 0.8-2.2 µg/100 g) that is comparable to the average content in commercial eggs determined in **Paper III**. It is also comparable to the value of 1.1 ± 0.2 µg/100 g measured in eggs from the control group in trial 1.

5.1.1.5 Trial 1

Design

The 40 hens were equally divided into four groups: three UVB treatment groups and one control group. The four groups were placed in each their cage. The doses received by the treatment groups were 19,000 J/m^2/day, 29,000 J/m^2/day and 35,000 J/m^2/day, respectively. The dose of 29,000 J/m^2/day (1.06 W/m^2) was obtained in 7 hours and 38 min per day (from 04:00-11:38). The dose of 35,000 J/m^2/day (0.76 W/m^2) was obtained with an exposure time of 11 hours and 47 min per day (from 03:00-15:47). An overview of the trial is displayed in Figure 5-5.

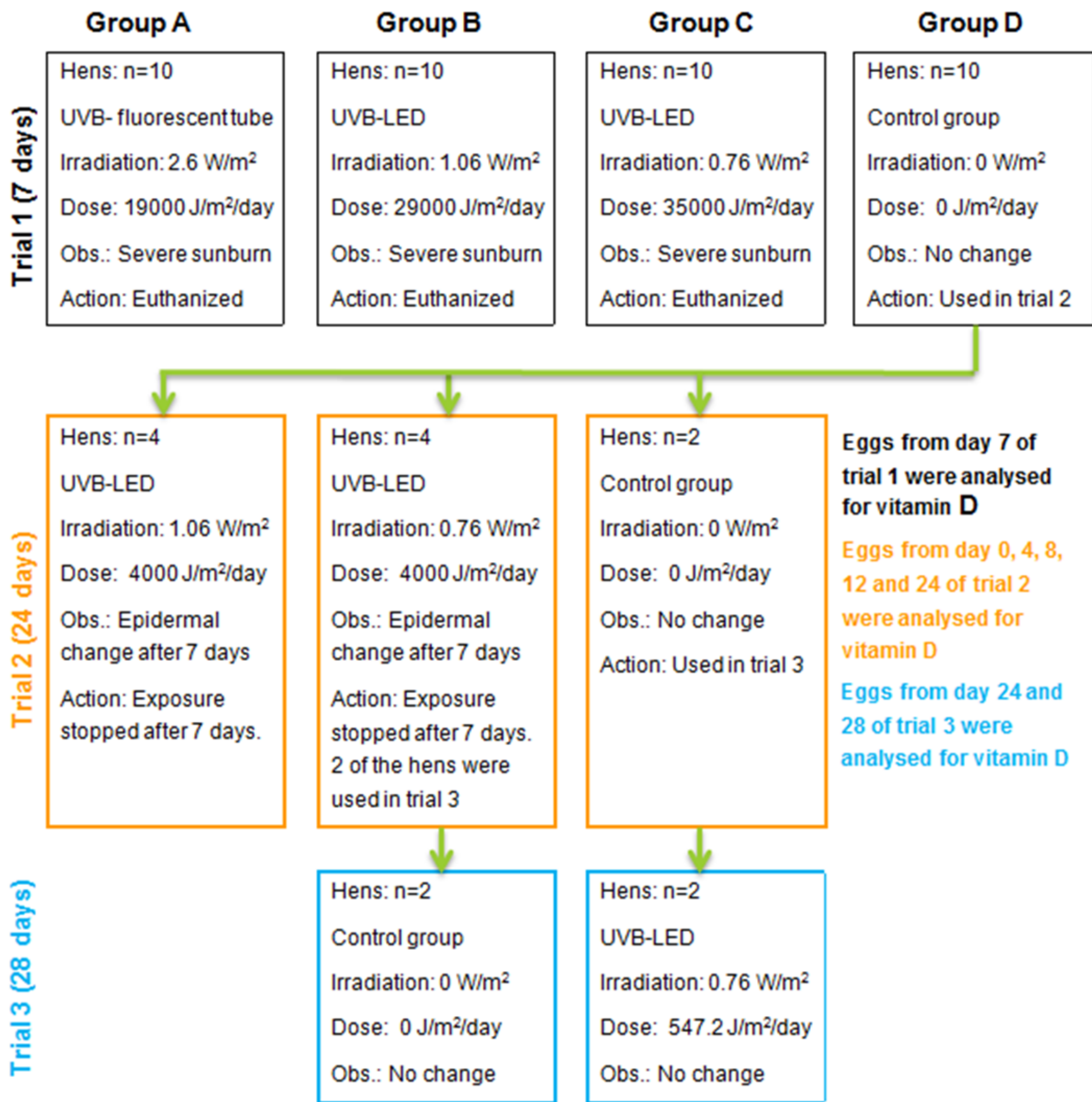


Figure 5-5 Overview of trial 1 (top box, black), 2 (middle box, orange) and 3 (bottom, blue). Each trial successively followed each other, 59 days in total. Information on the size of each group, light exposure, visual observation at the end of each trial, and action at the end of the trial is given in each box. To the right is listed which eggs were analysed for vitamin D. Notice that 2 hens from a UVB exposure group in trial 2 were used as control in trial 3 under the assumption that the vitamin D_3 content in the eggs would be back to baseline 18 days after ceased UVB exposure and therefore before trial 3 was started.

Sample collection

As it was originally planned to collect eggs weekly, only eggs from the last day of trial 1 were collected. 4 eggs from the control group and 6 eggs from each of the UVB exposed groups were randomly picked for analysis.

Results and discussion

After 7 days of exposure severe sunburn was observed on the comb and facial skin of all the hens in the UVB treatment groups. The trial was immediately stopped and the affected hens were euthanized. The content of 25(OH)D_3 and vitamin D_3 determined in the eggs is shown in Figure 5-6. There was no increase in the content of 25(OH)D_3 in eggs from the treatment groups compared to the control. An increase between the UVB treatment groups and the control was observed, there where however no difference between the LED-UVB treatments. Compared to the control group the LED-UVB (29,000-35,000 J/m^2/day) treatment increased the vitamin D_3 content of egg yolk with an average of 90 % and the UVB-tube (19,000 J/m^2/day) increased the content with 45 %. It was therefore show possible to increase the vitamin D content in eggs when exposing hens to UVB from above; however, the doses were way too high. The highest dose was approximately equal to the total dose of UVB on a summer day from 06:00 to 18:00 in Schauinsland, Germany (47 °N) (calculated by use of data from Urbain & Jakobsen (2015)). Regrettably, in retrospect it seems logical that such a high dose was too high as the hens had never before been exposed to sunlight and it is unknown if the hens could have tolerated the dose if an adaption period had been used.

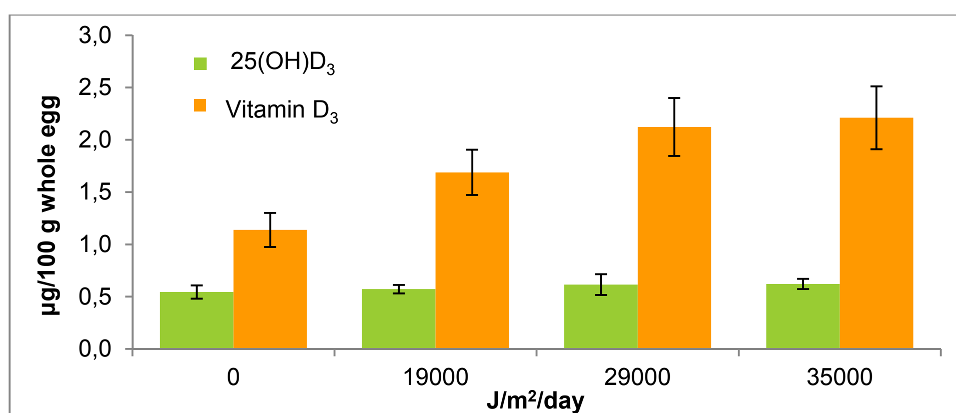


Figure 5-6 Content of vitamin D_3 and 25(OH)D_3 in eggs from hens exposed to different doses of LED-UVB for 7 days. The dose of 19,000 J/m^2/day was obtained using a UVB emitting fluorescent tube while the two higher doses were obtained using UVB-LED. The black bars indicate the SD.

5.1.1.6 Trial 2

Design

The 10 hens from the control group were split into three; two treatment groups with each 4 hens, and a control group with 2 hens. The dose was decreased significantly from the first trial in order to avoid sun burn. Both treatment groups received the same daily dose of 4000 $J/m^2/day$. This time the exposure was split into time intervals of 5 minutes spread evenly over the entire daylight period. Based on the results from trial 1 it was planned to increase the dose of one of the treatment groups gradually after one week. An overview of the trial is displayed in Figure 5-5.

Sample collection

Egg were collected on day 0, 4, 8 12 and 24 of trial 2. For each day of analysis all eggs from the treatment groups were analysed (3-4 eggs).

Results and discussion

After 7 days, before the dose had been increased, changes in the combs of the UVB exposed hens were observed; the combs were darker and they had a slight change in structure (more stiff) and the tips of the combs seemed lightly swollen and the UVB treatment was therefore stopped. The collection of eggs continued until 28 days after first UVB exposure in trial 2. In retrospect the observed changes of the comb could be a normal, unharmful, epidermal response to UVB exposure when comparing it to the combs of free-range hens; however, due to the fatal outcome of trial 1, extra caution was taken at that time.

There was no difference in the vitamin D_3 content in eggs from the two exposure groups, which had been exposed to the same dose of 4000 $J/m^2/day$ using two different total irradiances (0.76 and 1.06 W/m^2); this is consistent with the results presented in Section 4.4, which showed that the production of vitamin D only depended on dose and not total irradiance. The eggs collected on day 12 (5 days after ceased exposure) showed a tendency to have a higher content of vitamin D_3 than the eggs collected on day 7 (the last day of exposure); indicating that the content of vitamin D continued to increase after ceased exposure (see Figure 5-7). However, the content was back to baseline 8 days after the treatment was stopped (on day 16). Baseline was reached approximately 15 days after withdrawal in a study where hens were had been fed with vitamin D_2 in the form of UVB exposed shitake for 28 days; however, before

withdrawal the eggs had a vitamin D_2 content of ~12 μg/100 g (Kawazoe et al. 1994). The fact that the content was 7 times higher in the feeding trial might explain the longer time to reach baseline compared to the observations in the present trial.
The content of 25(OH)D_3 remained unchanged with a value of 0.6 ± 0.09 μg/100 g.

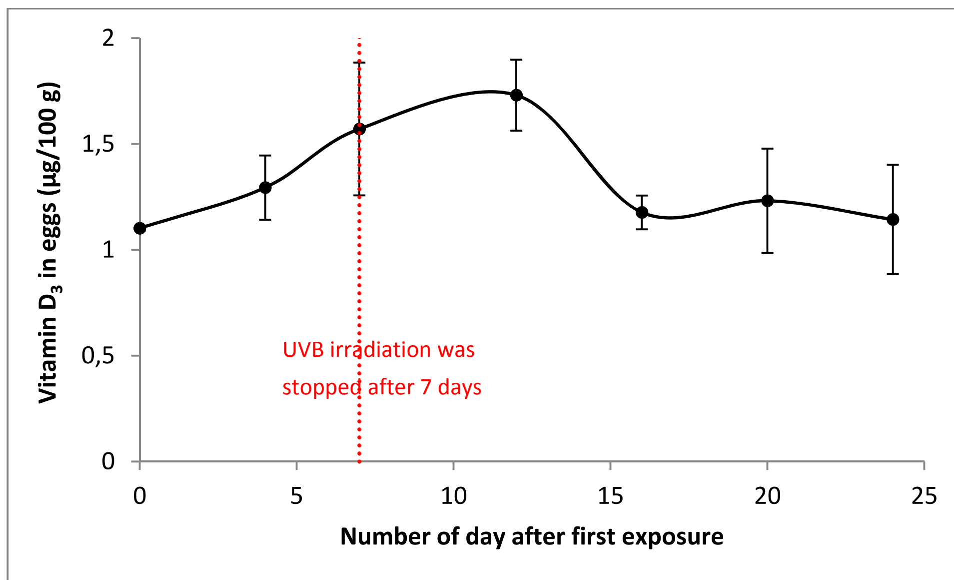


Figure 5-7 Results from trial 3 where 8 hens were exposed to a UVB dose of 4000 J/m^2/day. The exposure was stopped after 7 days of exposure due to observed redness of the combs. The vitamin D_3 content in whole eggs (± SD) as a function of number of days after first exposure is displayed.

5.1.1.7 Trial 3

Design

The two hens from the control group from trial 2 became the new treatment group, and 2 of the hens from the treatment groups became the control as it was assumed that the vitamin D level in the eggs were back to normal (according to the results from trial 2) as trial 3 was started 18 days after the UVB exposure of trial 2 was stopped. The UVB treatment group was exposed to 547.2 J/m^2/day for 28 days. The exposure was split into time intervals of 1 min spread evenly over the entire daylight period; 12 min in total. An overview of the trial is displayed in Figure 5-5.

Sample collection

All eggs were collected each day; 2 per day per treatment. The eggs were kept as whole at 5 °C until analysis.

Results and discussion

No signs of epidermal response was observed during trial 3.

The content of vitamin D_3 and 25(OH)D_3 were measured in the egg yolks after 24 and 28 days of exposure. All eggs from both the treatment and the control group were analysed (2 per day per treatment). No difference in either 25(OH)D_3 or vitamin D_3 content was observed between the control and the treatment group (data not shown). Lietzow et al. (2012) used the same dose but UVB emitting fluorescent tubes (280-310 nm) instead to expose hens from above and found no effect. The hypothesis of trial 3 was that by using a more optimal wavelength for vitamin D production the dose would be effective in increasing the vitamin D_3 content in eggs. However, the dose were simply too low to get a response, optimal wavelength or not.

5.1.2 Direct UVB irradiation of liquid egg products

The content of 7-DHC in egg yolk is around 140 µg/100 g (Kühn et al. 2014) and this could in theory be converted to vitamin D_3 by exposing liquid egg products (whole eggs or yolks) directly to UVB. A small pilot study was set up, where both whole eggs and egg yolk were exposed directly to UVB. The samples were homogenised using a hand mixer before transferring about 3 grams to 2x2 square trays. The layer thickness was 3-4 mm. The UVB LED with a central wavelength of 296 nm was used (see Table 4-2) to expose either liquid whole egg or yolk to two doses of UVB: 300 J/m^2 and 3000 J/m^2 (see Figure 5-8 for a picture of the experimental setup). The exposures were performed in duplicate, however the analyses of one the whole egg samples failed.

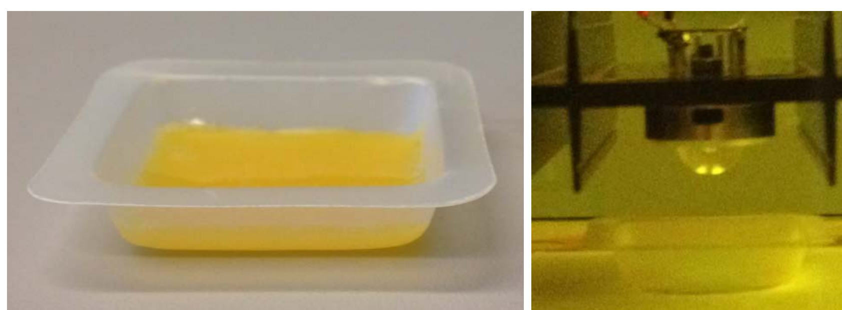


Figure 5-8 Experimental setup of direct exposure of egg yolk. **Left:** Homogenised egg yolk was placed in a layer of 3-4 mm in a plastic weighing boat. **Right:** Placement of the weighing boat below the UVB LED.

The content of vitamin D_3 was determined using the HPLC-UV-DAD method described in Section 3.3 and the results are displayed in Table 5-1. With a dose of 3000 J/m^2 the

vitamin D_3 content was increased approximate 4 times in both liquid egg products. In this setup it took 3.5 minutes to deliver the dose of 3000 J/m^2, however the dose can be adjusted by changing the exposure time and the total irradiance as described in Section 4.4 and the vitamin D_3 content can thereby be tailored.

Table 5-1 Content of vitamin D_3 measured in liquid egg products after direct exposure to UVB at 296 nm.

Dose (J/m^2)	Vitamin D_3 in liquid egg product (µg/100 g)	
	Whole egg	**Yolk**
0	1.1	5.2
300	2.0	7.6
	2.6	7.5
3000	4.3	20.7
	-	17.1

5.2 Vitamin D enhanced pork products

5.2.1 Description of the UVB emitting fluorescent tubes used in the pig trial (Paper II)

Due to the high costs of UVB LED it was not possible to use it in the pig trial. Instead UVB emitting fluorescent tubes intended for reptiles were used; the tubes had a central wavelength of 312 nm in the UVB region. It did however also include other visible wavelengths as seen from the full spectrum displayed in Figure 5-9. The characterisation of the tubes was performed by DTU Fotonik as described in **Paper II**.

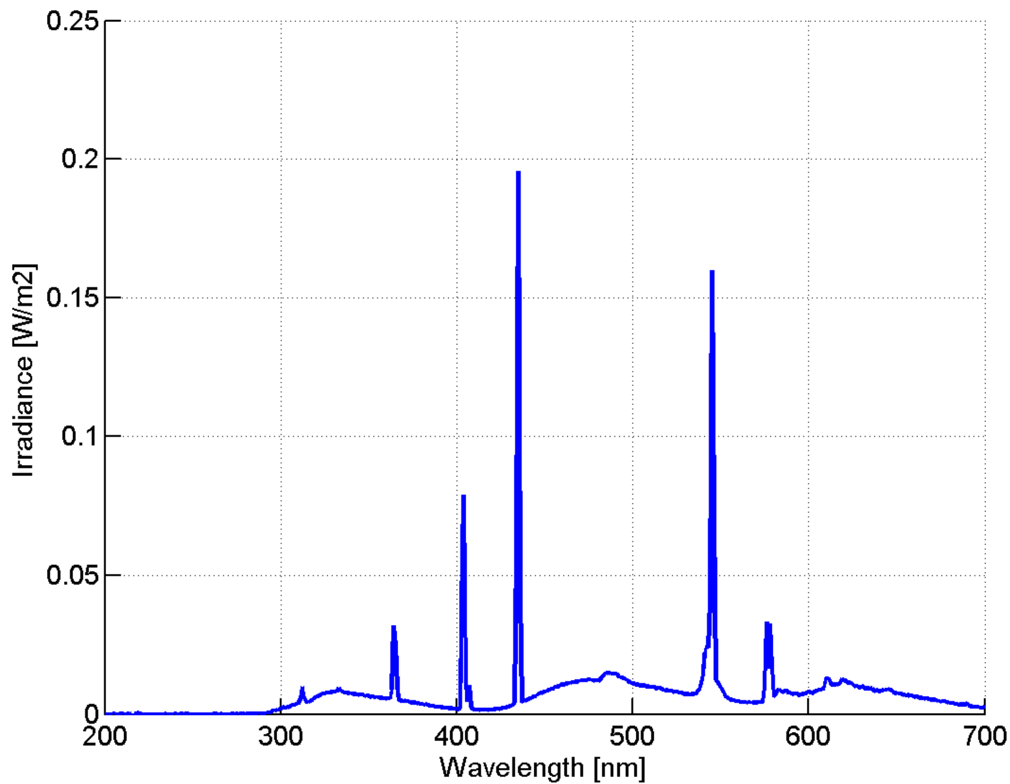


Figure 5-9 The full spectrum of the fluorescent tube, used for the UVB exposure of the pig, measured at a distance of 70 cm (from **Pape II** with permission).

With the need for changing the UVB source and the knowledge that the vitamin D_3 production does not only depend on the dose of UVB but also on the wavelength (see Section 4.2) it was realised that in order to compare results across studies reporting the dose would not be enough, the dose had to be standardised according to wavelength. It was decided to use the erythema reference spectrum in order report the dose in units of standard erythemal dose (SED); 1 SED equals 100 J/m^2 erythemal dose. The erythema reference spectrum shows the ability of specific spectral wavelengths to induce erythema in human skin normalised to the maximum (International Commission on Illumination 1999). The total irradiation in the measured UVB spectrum was weighted against the erythema reference spectrum. The UVB part of the measured spectrum overlaid with the erythema reference spectrum is displayed in Figure 5-10. Expressing the dose as SED had the advantage that it was possible to directly compare doses to a study where mini-pigs had been exposed to artificial UVB (Burild et al. 2015); the mini-pigs were exposed with up to 1.8 SED, and no erythema had been observed.

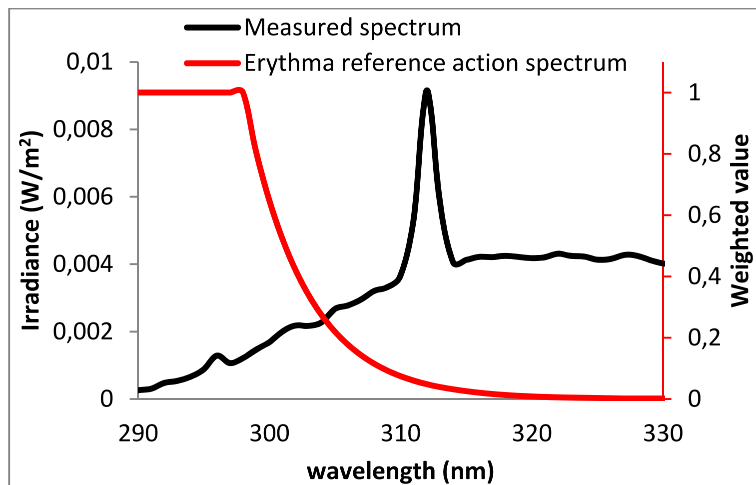


Figure 5-10 The UVB part of the full spectrum displayed in **Figure 5-9** overlaid with the erythema reference action spectrum superimposed (from **Paper II** with permission).

5.2.2 UVB irradiation of pigs (**Paper II**)

In the pig trial 24 slaughter pigs were divided into 4 groups: 1 control and 3 UVB treatment groups. The pigs were kept at the research facility for large animals at University of Copenhagen (Rørrendegård, Taastrup, Denmark). Solid walls were put up between the pens to avoid UVB pollution between the groups as shown in Figure 5-11.

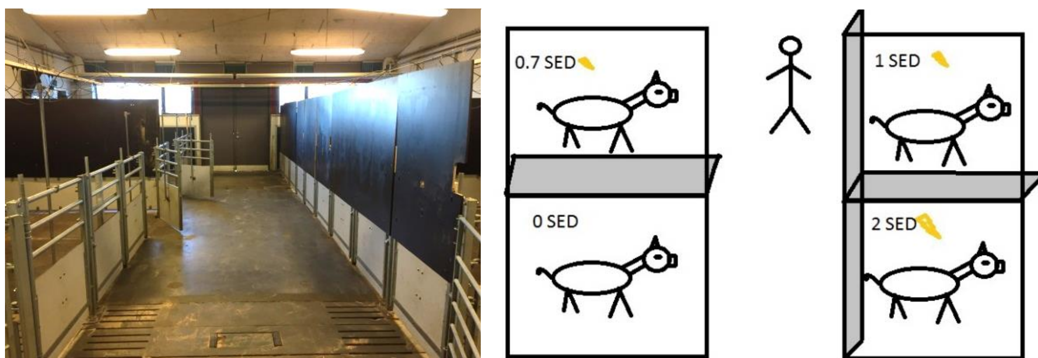


Figure 5-11 The pens with solid walls installed (**left**) and a graphic overview (**right**)

4 UVB emitting fluorescent tubes (described in section 5.2.1) were placed in each of the treatment pens at a height of approximately 180 cm above the floor as indicated by the orange arrow in Figure 5-12.

Figure 5-12 Picture of a pen where one of the four UVB emitting fluorescent tubes installed in each pen can be seen (indicated by the orange arrow).

The three treatment groups were exposed to the target doses of 0.7, 1 and 2 SED/day after a 7 day adaption period where the dose was gradually increased. The exposure period ran 28 days in total. However, at day 19, the 2 SED treatment had to be stopped as mild erythema was observed on the ears and backs of the pigs. Therefore, only results from the three remaining groups are presented. A graphic overview of the timeline of the trial is displayed in Figure 5-13.

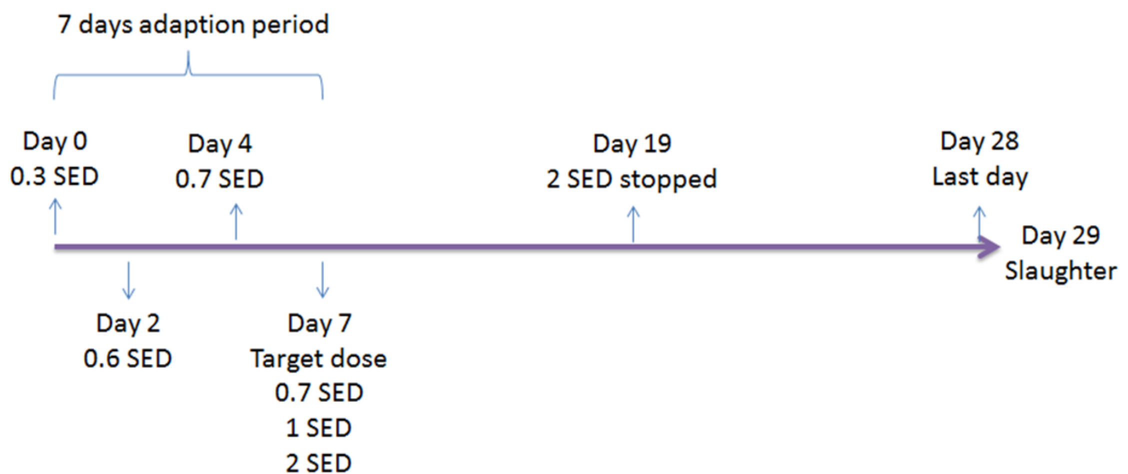


Figure 5-13 Graphic illustration of the timeline of the pig trial. The pigs were first adapted to UVB and reached their target doses on day 7. The pigs were exposed to UVB for a total of 28 days expect the 2 SED group where the treatment was stopped at day 19 due to mild erythema. The pigs were slaughtered on day 29.

The pigs were slaughtered on day 29, the day after the last exposure. Serum samples were collected during slaughter. The rind of the pigs was treated as conventional slaughter pig as they were scalded and dehaired in a scalding tub and then singed; the black deposits and singed hairs were scraped off with a knife and finally washed off. The carcasses were hung in cold storage overnight. The next day the middle part of the pork loin was sampled from each left side of the pigs. The pork loin was divided into rind, subcutaneous fat and lean meat as indicated by the orange arrows in Figure 5-14, the leftovers were discarded. Subcutaneous fat, lean meat and liver were homogenised while a representative sample from the rinds were obtained by stratified randomisation as illustrated in Figure 5-14.

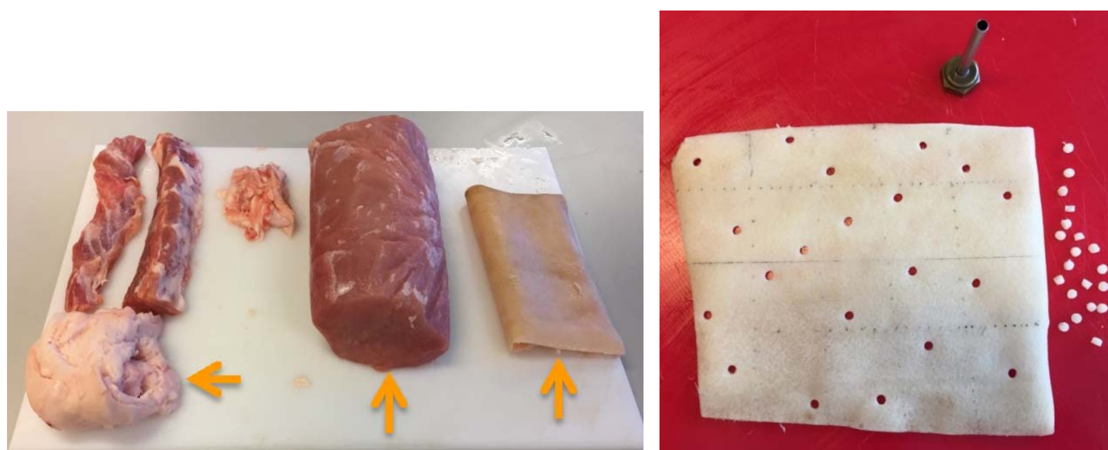


Figure 5-14 Pork loin was divided into subcutaneous fat, lean meat and rind (indicated by orange arrows). The remains that did not belong to either of the pure cuts were discarded. The picture to the left shows how a representative sample from rind was collected by stratified randomisation where the square pattern is the stratification.

The highest content of $25(OH)D_3$ and vitamin D_3 was obtained with 1 SED (see Figure 5-15) where the total vitamin D content in rind, subcutaneous fat, lean meat and liver was 33.7 µg/100 g, 12.7 µg/100 g, 0.61 µg/100 g, and 3.7 µg/100 g, respectively.

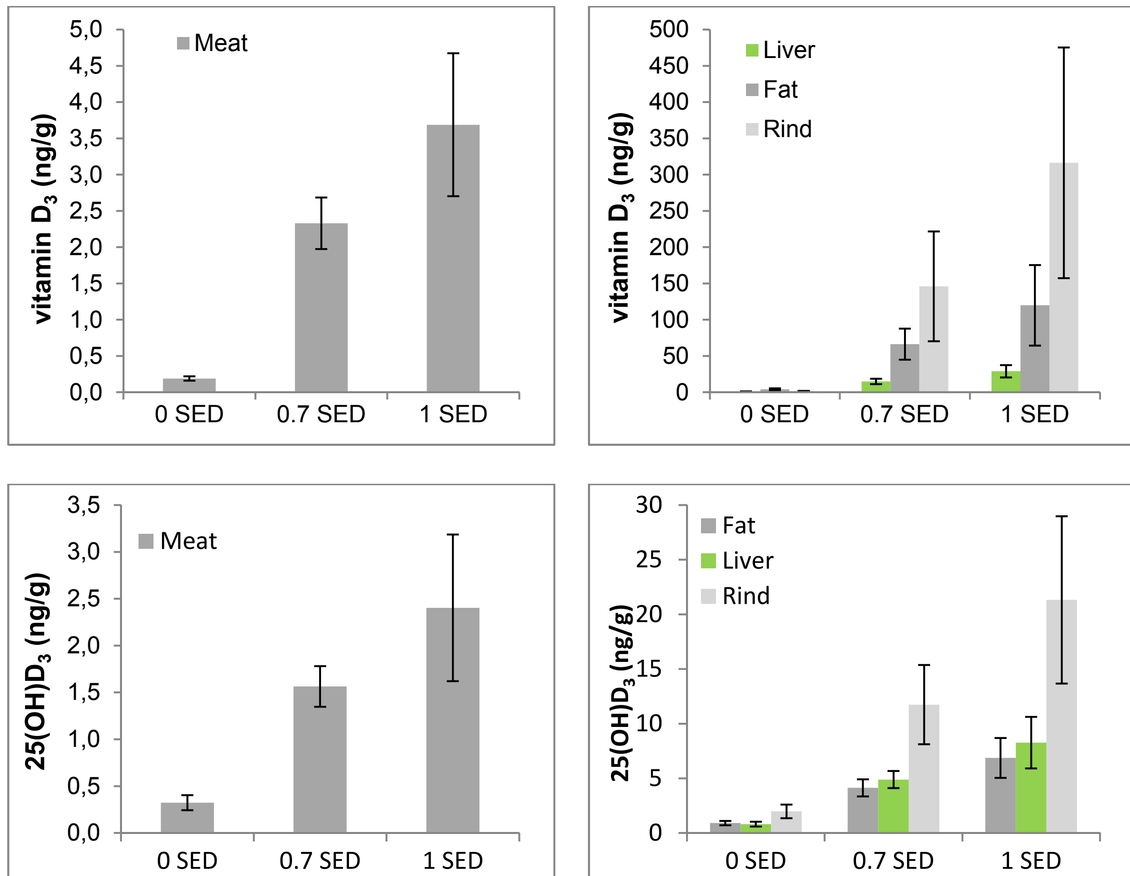


Figure 5-15 Content of vitamin D_3 and $25(OH)D_3$ in rind, subcutaneous fat, lean meat and liver after pig have been exposed to different doses of UVB for 28 days. The black bars indicate the SD.

A linear association between dose and vitamin D_3 content was found, which indicated that the maximum production was presumably not reached as others have found that the serum $25(OH)D_3$ in humans increase linearly with dose up to approximately 25 SED from whereon the response flattens (based on 18 studies reviewed by Grigalavicius et al. (2015) and a linar association with doses from 0.375 SED to 3 SED has also been reported in humans (Bogh et al. 2011). The serum $25(OH)D_3$ continued to increase for approximately 50 days in mini-pigs when exposed to UVB (Burild et al. 2015); therefore, increasing the exposure period beyond 28 days could also possible result in higher content of vitamin D. As the maximum was not reached the potential of the method was illustrated by using the pig with the highest content of vitamin D to calculate the potential content in two pork products: the total vitamin D activity in 100 g of minced meat with 10% fat was calculated to be 3.7 µg/100 g, and in a traditional Danish liver paté (35% liver and 15% lard) the activity was calculated to be 2.1 µg/100

g. Products with contents ≥ 1.5 µg/100 g can be labelled ‘high content of vitamin D’ according to European legislation (Regulation (EC) No 1924/2006).
Use of a higher dose could possibly increase the content further; however 2 SED caused erythema after 19 days but if a longer adaption period had been used this might not have happened. Mini-pigs does not develop erythema when they are exposed to a UVB dose of 0.9 SED for 84 days followed by 1.8 SED for 35 days (Burild et al. 2015). A third option is to use the optimal wavelength (296 nm, see section 4.2) for vitamin D instead of the 312 nm emitted by the used fluorescent tubes, as the production at 296 nm is approximately 10 times more effective. To illustrate the potential the measured spectrum was parallel shifted to have its maximum at 296 nm (see Figure 5-16) and then the erythemal effective irradiance was calculated to be increased by a factor of 3. Keeping the dose of 1 SED constant would decrease the exposure time to 1/3 but the content of vitamin D_3 would be increase approximately 3 times.

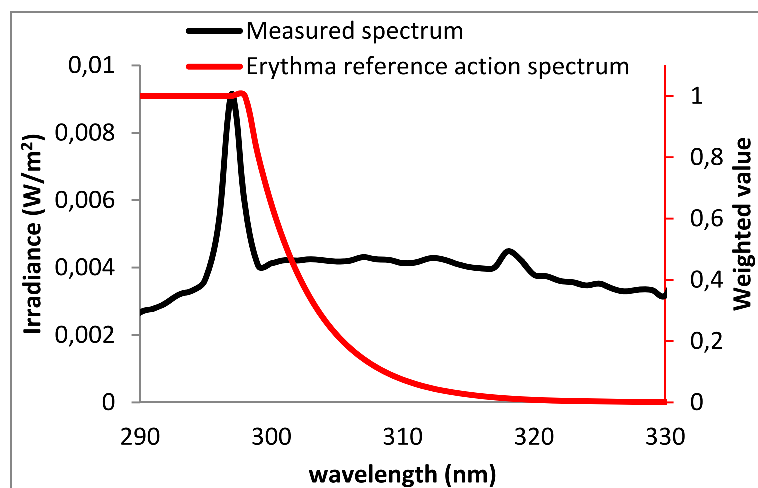


Figure 5-16 Parallel shifting of the UVB spectrum shown in **Figure 5-10** to have its maximum at 296 nm. The erythema reference action spectrum is superimposed.

There were a high within group variation especially in the vitamin D_3 and $25(OH)D_3$ content in rind from the exposed groups, see Table 5-2. The CV % of serum $25(OH)D_3$ varied across the groups independent of UVB exposure. In humans the CV % shows a tendency to increases with UVB dose; when not exposed the CV % is ~ 21 and the highest CV % after exposure is 33 % (Datta et al. 2016); except for the tendency of the CV % to increase with dose these results resembles the CV % of 15-27 observed for the pigs (see Table 5-2).

Table 5-2 CV% of the mean of the measured content of vitamin D_3 and 25(OH)D_3 in the different parts analysed.

		CV %		
		0 SED	**0.7 SED**	**1 SED**
Rind	Vitamin D_3	12	52	50
	25(OH)D_3	50	33	38
Subcutaneous fat	Vitamin D_3	27	32	47
	25(OH)D_3	22	20	26
Lean meat	Vitamin D_3	15	17	27
	25(OH)D_3	27	13	33
Liver	Vitamin D_3	38	26	31
	25(OH)D_3	25	16	29
Serum	Vitamin D_3	38	24	29
	25(OH)D_3	27	15	26

A linear relationship was found between serum 25(OH)D_3 and vitamin D_3 and 25(OH)D_3 content in all cuts. The Pearson's r values from all the correlations is given in Table 5-3 and as an example the correlation is plotted for lean meat in Figure 5-17.

Table 5-3 Linear association between serum 25(OH)D_3 and vitamin D_3/25(OH)D_3 content in other part of the pig. From **Paper II** with permission.

	Pearson's R	
Part of the pig	**Vitamin D_3**	**25(OH)D_3**
Rind	0.92	0.97
Subcutaneous fat	0.95	0.95
Lean meat	0.96	0.97
Liver	0.98	0.98
Serum	0.96	-

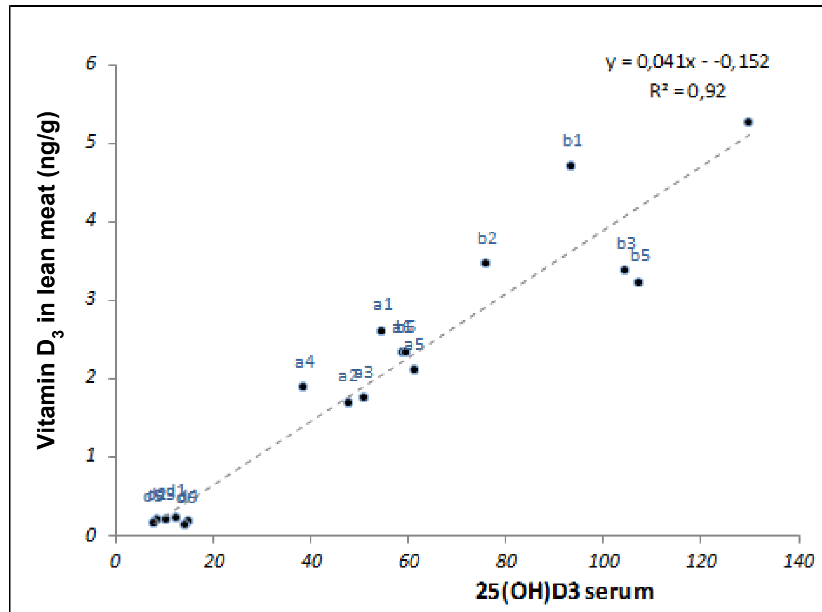


Figure 5-17 Correlation between serum 25(OH)D_3 and vitamin D_3 content in lean meat from pork loin from individual pigs. The letters indicate UVB treatment group: **d** 0 SED; **a** 0.7 SED; **b** 1 SED. The superscript numbers indicate the specific pig.

5.3 Discussion

5.3.1 Choice of UVB source

If narrowband UVB with wavelengths below ≤ 300 nm is used the major photodegradation product is changed from being L_3 to being T_3 compared to UVB sources with a broadband spectrum (see Section 2.6). A T_2 content ≤ 0.93µg/100g in the final food is considered safe by EFSA (EFSA NDA Panel 2014). L_3 is the major photodegradation product in skin but it is not expected to be of any health issue as the toxic dose of L_2 in rats is above 2000 µg (Bekemeier & Pfordte 1963). At doses ≤ 4375 J/m^2 UVB (290-320 nm) PreD_3 is the main product in skin and T_3 and L_3 is not detected (Holick et al. 1981). The doses used in trial 2 of the hens, in direct exposure of the liquid egg products and in the pig trial were all below 4375 J/m^2 and it is therefore anticipated that the production of L_3 and T_3 was minimal although it was not determined.

Exposure to UVB might have health benefits for the animals as well; currently a research project is running where sows and piglets are to be exposed to UVB using LED in order to bring down the mortality of the piglets and improve the health of the sows (The Danish Environmental Protection Agency n.d.). In the study with pigs presented in this thesis UVB emitting fluorescent tubes were used, but they are not

feasible in an industrial pig production as they have high energy consumption; LED is the future as they have longer expected lifetimes and lower maintenance costs and UVB LED is under development (Oh et al. 2019; Khan et al. 2019). The UVB LEDs under development have wavelengths optimal for vitamin D production and do not resemble the UVB output from the sun; however, as pointed out by Holick & Hossein-Nezhad (2017) exposure to the whole light spectrum of the sun might have other health benefits as well, whereas narrow-band UVB only is good for vitamin D production.

5.3.2 Effectiveness of UVB exposure compared to feeding vitamin D for the enhancement of eggs (**Paper III**)

In **Paper III** the effect of adding vitamin D_3, 25(OH)D_3 or a combination of the two to feed was reviewed. An inter-trial linear relationship between vitamin D_3 in feed and vitamin D_3 content in eggs was found (see Figure 5-18).

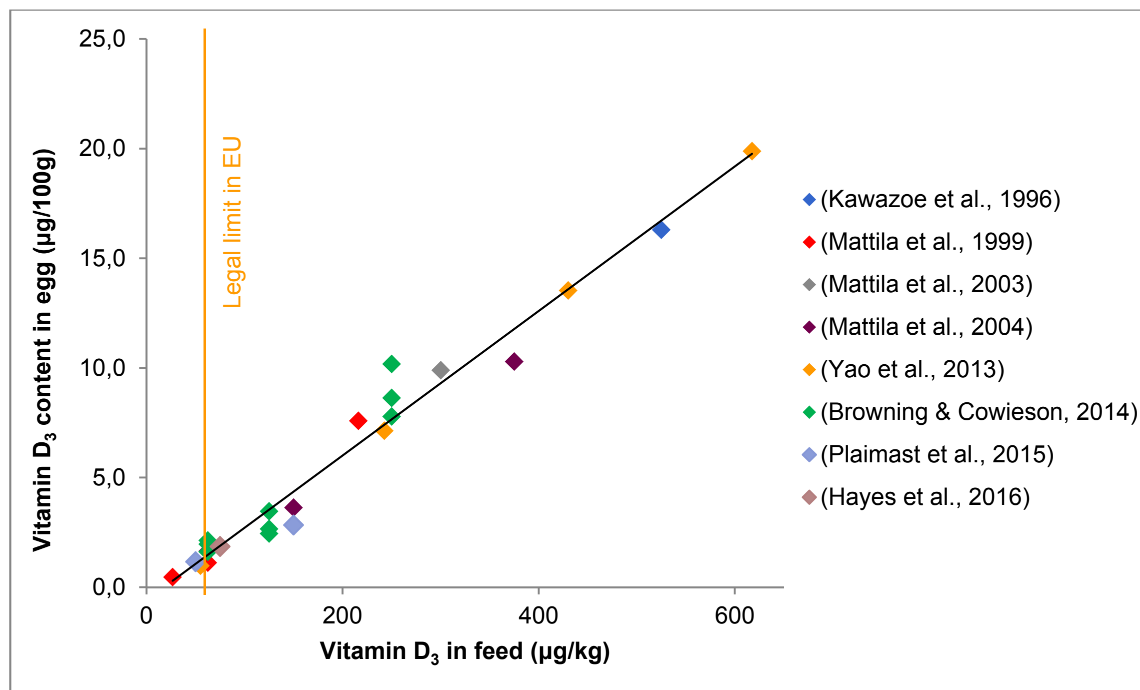


Figure 5-18 Content of vitamin D_3 in eggs as a function of vitamin D_3 in feed based on published data as referenced in the figure.

The equation for the linear relationship is:

$$\text{Vitamin } D_3 \text{ in eggs } (\mu g/100g) = 0.033 \cdot \text{vitamin } D_3 \text{ in feed } (\mu g/kg) - 0.58)$$

Pearson correlation coefficient (Pearson's r) for the equation is 0.987 and the linear relationship was statistically significant with a p-value of $6 \cdot 10^{-19}$ (F-test) in spite of the

differences in the design of the studies described in **Paper III**. The 25(OH)D_3 content in eggs did not respond in the same clear inter-trial manner; however, it was estimated that the relationship was logarithmic rather than linear, with a maximum around 1 µg/100 g, within reasonable feeding levels.

When feeding 25(OH)D_3 alone or in combination with vitamin D_3 the content of 25(OH)D_3 in the eggs will increase; there is however no clear inter-trial relationship. 25(OH)D_3 is less effective in increasing the total vitamin D content than feeding vitamin D_3. Supplementing hens with 122 µg 25(OH)D_3/kg feed gives eggs with 1.4 µg 25(OH)D_3/100 g (Mattila et al. 2011) whereas from the equation given above it is estimated that the same level vitamin D_3 would result in 3.4 µg vitamin D_3/100 g. Feeding 69 µg 25(OH)D_3 in combination with 250 µg vitamin D_3 per kg feed gives eggs with a total vitamin D content of 12.9 µg/100 g (Browning & Cowieson 2014) to obtain the same amount by feeding pure vitamin D_3 would according to the equation, under the assumption that the 25(OH)D_3 content is 1 µg/100 g, require approximately 378 µg/kg feed.

No negative health effects has been found of feeding 300 to 2555 µg vitamin D_3 /kg (Persia et al. 2013; Mattila et al. 2004; Mattila et al. 2003) whereas negative effects have been observed when feeding 413 µg 25(OH)D_3 per kg feed (Terry et al. 1999). Schutkowski et al. (2013) and Kühn et al. (2015) enhanced eggs with vitamin D by using UVB exposure directed at the feet of the hens; however, implementing UVB-lamps directed at the feet of the hens in a barn will be a challenging task. If placed near the floor the lamps will get dirty, and a vast amount of cleaning will be required. In addition only the hens nearby the lamp will be exposed, making it hard to control the dose each hen receives. If UVB-treatment is to be implemented in barn egg production facilities it will be necessary to develop solutions where the hens are exposed from above, in order to meet future needs. This way an even exposure would be secured and minimisation of cost would be achieved both for maintenance and cleaning. It was shown possible to increase the vitamin D content in eggs from hens, supplemented with 75 µg vitamin D_3/kg feed, by exposing them to artificial UVB from above at a wavelength of 306.5 nm. However, an appropriate dose was not determined. A dose at or above 19,000 J/m^2/day was much too high and resulted in severe sunburn after 7 days of exposure. With a dose of 4000 J/m^2/day, changes of the combs were observed after 7 days. In retrospect the observed change could be a normal, unharmful, epidermal response to UVB-exposure when comparing it to the combs of free-range hens. It is therefore believed that with further research it will be possible to find a dose

that will increase the vitamin D_3 content of eggs, without causing erythema in the hens. It could possibly be that the hens, which have never been exposed to UVB before need an adaption period, where the dose of UVB is gradually increased.
It has been shown that the vitamin D_3 content in eggs increases non-linearly with UVB dose, and that the total vitamin D content at equilibrium is most likely below 6 µg/100 g according to the findings of Schutkowski et al. (2013) and Kühn et al.(2015). The same amount could be achieved by supplementing hens with 169 µg vitamin D_3 /kg feed and it could be further increased. It is therefore suggested that enhancing eggs through feed is the best choice, as it can be increased above what might be possible with UVB, and it only requires a change in the feed composition, and therefore has no upfront costs or cleaning/maintenance expenses.

5.3.2.1 European legislation regarding vitamin D in feed for hens

In Europe there are maximum limits for the addition of vitamin D to feed. It is allowed to feed both laying hens and pigs with vitamin D_3, 25(OH)D_3 or a combination, as long as the total content is ≤ 80 µg/kg feed for laying hens and ≤ 50 µg/kg feed for pigs (Commission Regulation (EU) 2017/1492, Commission Regulation (EC) No 887/2009)
In Commission Regulation (EU) 2017/1492 it is stated that the current maximum limit, of 80 µg/kg feed for laying hens, does not have adverse effects on animal health, human health or the environment and that the limit is based on scientific opinions from the European Food Safety Authority (EFSA). The reasoning behind EFSAs conclusion regarding animal safety was that this level has been used for over a decade without any reported intolerances; they were however unable to draw any final conclusion as their answer was solely based on data from the National Research Council (NRC) from 1987. In regard to human health, they conclude that since there has been no change in the level of vitamin D in feed during the last decade it is safe to continue with the same level (EFSA 2014). The fact that the human intake of vitamin D is below the recommended was not taken into consideration.
One can apply for changing the limits by sending an application to the European Commission whereby EFSA shall make a new scientific opinion. If the opinion is positive the Standing Committee on Plant, Animals, Food and Feed (PAFF committee - section animal nutrition) will discuss, draft and possibly approve a new regulation according to the procedure described in Regulation (EC) No 1831/2003. As an example the Norwegian Food Safety Authority (NFSA) applied for increasing the maximum limit 20 times, from 75 µg/kg feed to 1.5 mg/kg feed for salmonids and

received a positive opinion from EFSA (EFSA 2017; EFSA 2019). The opinion is currently being discussed at PAFF committee meetings (Standing Committee on Plant, Animals 2019).

5.3.3 Possible dual effect of UVB when used for direct exposure of liquid egg products

It was shown possible to produce vitamin D_3 enhanced liquid egg products (yolk and whole egg mix) by direct exposure to UVB. The penetration depth of UVB at 296 nm is 0.01-0.08 mm (Argyraki 2017); therefore, any practical application would have to be on a very thin film or in a dynamic treatment (e.g. stirring). This process could benefit from the dual effect of UVB as it has been shown that UVB is more effective than UVC in inactivating the bacteria *Pseudomonas aeruginosa* in a trial where the exact same UV-LED as used for UVB exposure of hens was used; a dose of 10,000 J/m^2 left no viable colonies, and the exposure time was less than 12 minutes. Dynamic treatment of liquid egg products with a UVC dose of 42,000 J/m^2 obtained in 30 minutes has been shown to have the same disinfectant properties as heat pasteurization but with only minor changes to the rheological properties (de Souza & Fernández 2013). Therefore, there is potential that a dynamic treatment with UVB, alone or in combination with UVC could produce both safe and vitamin D enriched liquid egg products. Narrowband UVB would probably be used for such application with the consequence that the distribution between the photodegradation products would change depending on the chosen wavelength (MacLaughlin et al. 1982); it will therefore be necessary to have analytical methods that include at least L_3 and T_3 in the analysis although the production is expected to be minimal if doses below 4375 J/m^2 is used (Holick et al. 1981).

5.3.4 Novel foods regulation in the EU

Food from animals exposed to artificial UVB light or food directly exposed to artificial UVB will most likely have to be approved under the novel foods act in Europe (Regulation (EC) 2015/2283); as a novel food is defined as a food that has not been consumed by humans in EU before 1997.

For an application to be made, more detailed analysis including T_3, L_3 and $preD_3$ will probably have to be made; especially if narrowband UVB with wavelengths ≤ 300 nm is used (see Section 5.3.1).

6. Conclusion

The main aim of this thesis was to show that it was possible to produce vitamin D enhanced eggs and pork by exposing hens and pigs to UVB in a way that could eventually be implemented in industrial settings. Such investigations require sensitive, reliable and specific analytical methods in order to quantify the vitamin D active compounds, vitamin D and 25(OH)D.

Preliminary invetigations where minipig skin was exposed to UVB *ex vivo* showed that the optimal wavelenght for vitamin D production in skin was 296 nm; and that the vitamin D_3 production depended on the dose and not the exposure time or total irradiance.

It was shown that the vitamin D_3 content in eggs could be increased by exposing encaged hens to UVB from above; however, changes in the colour and structure of the combs were observed after 7 days of exposure to 4000 J/m^2/day and the treatment was stopped. In hindsight the observed change might have been a natural harmless response when comparing it to hens kept outdoors. The highest content of vitamin D_3 in the eggs was measured 5 days after ceased exposure, where the total vitamin D content was 2.1 µg/100 g in whole egg. According to the findings of Schutkowski et al. (2013) and Kühn et al. (2015) the vitamin D_3 content in eggs increases non-linearly with UVB dose and the total vitamin D content at equilibrium is most likely below 6 µg/100 g. Based on existing published data a linear response between vitamin D_3 in feed and vitamin D_3 content in eggs was shown. 6 µg/100 g could be achieved by supplementing hens with 169 µg vitamin D_3 /kg feed. The content could be further increased as there has not been observed negative health effects of feeding 300 to 2555 µg vitamin D_3/kg (Persia et al. 2013; Mattila et al. 2004; Mattila et al. 2003). It was therefore concluded that feeding vitamin D_3 is more effective than UVB exposure to increase the vitamin D content in eggs.

UVB emitting fluorescent tubes were installed in settings comparable to a conventional pig farm. With a dose of 1 SED/day for 28 days (including 7 days adaption period) before slaughter the total vitamin D_3 content in lean meat and subcutaneous fat was a

factor 19 and 29 higher, respectively, compared to the control; while $25(OH)D_3$ was a factor 8 higher in both compared to the control.

It was also shown that it is possible to produce vitamin D_3 enhanced pork products by direct exposure of the product to UVB, if the product contains rind. Likewise it was shown possible to produce vitamin D_3 enhanced liquid egg products (whole egg and yolk) by direct exposure to UVB. The vitamin D_3 content of such products could be tailored by adjusting the dose of UVB.

Continuous post-column infusion of vitamin D_3-d_6 was used to visualise ion suppression in the analysis of vitamin D_3 as no blank matrix was available; by using this method it was possible to visualise the effect different eluent additives had on ion suppression in extracts of different food matrices. With a sample preparation consisting of saponification, liquid-liquid extraction, SPE and derivatisation with PTAD an enormous amount of ion suppression of vitamin D_3 was observed. Due to its earlier retention time vitamin D_3-d_6 could not fully eliminate ME in most food matrices.

7. Perspectives

Products with vitamin D contents ≥ 0.75 µg/100 g can be labelled ‘source of vitamin D’ and ≥ 1.5 µg/100 g can be claimed to have a ‘high’ content of vitamin D according to Regulation (EC) No 1924/2006. It was shown that the vitamin D_3 content in eggs and pork could be increased above these limits either by three method: by exposing hens or pigs to UVB; by exposing liquid egg products or rind directly to UVB; or by increasing the vitamin D_3 content in feed for layers. However, all three methods call for further research. There is need for long term trials in pigs; and the direct exposure of liquid eggs was only a small pilot trial; further research in dose-response of vitamin D and disinfection properties is needed. In addition, reliable analytical methods to determine the photodegradationproducts need to be developed.

In this thesis UVB emitting fluorescent tubes were used, but they are not feasible in an industrial pig production as they have high energy consumption; LED is the future as they have longer expected lifetimes and lower maintenance costs. UVB LED optimal for vitamin D production is under development (Oh et al. 2019; Khan et al. 2019). Exposure to the whole light spectrum of the sun might have other health benefits as well (Holick & Hossein-Nezhad 2017). For this reason it might be an idea to develop LEDs that resemble sunlight.

All the production methods presented in this thesis (both increased content in feed and UVB exposure) will require approval in the EU before they can be implemented in industry (see section 5.3.2 and 5.3.4).

The official method for determination of vitamin D in foods uses vitamin D-d_6 as IS and 0.1% formic acid as eluent additive (Gill et al. 2016). The use of vitamin D-d_6 in combination with formic acid as eluent additive could potentially lead to bias in some food matrices according to the findings presented in Section 3.4. It should therefore be investigated if the use of a ^{13}C labelled IS could correct for this.

PreD is less active than vitamin D (see Section 2.8, needs to be confirmed in humans). The inherent content of preD in foods is unknown; therefore, it is necessary to develop a method than can quantify preD and vitamin D separately in foods.

References

Alexander, B.M. et al., 2017. Sunlight exposure increases vitamin D sufficiency in growing pigs fed a diet formulated to exceed requirements. *Domestic Animal Endocrinology*, 59, pp.37–43.

Andersen, R. et al., 2013. Seasonal changes in vitamin D status among Danish adolescent girls and elderly women: the influence of sun exposure and vitamin D intake. *European Journal of Clinical Nutrition*, 67(3), pp.270–274.

Argyraki, A., 2017. *New light Sources for Biomedical Applications*. Technical University of Denmark (DTU). Available at: http://orbit.dtu.dk/en/publications/new-light-sources-for-biomedical-applications(4c1e9ea9-6508-4134-ba60-00fd50d1f00d).html.

Aronov, P.A. et al., 2008. Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 391, pp.1917–1930.

Barnkob, L.L. et al., 2019. Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities. *European Food Research and Technology*, 245(2), pp.411–418.

Bekemeier, H. & Pfordte, K., 1963. Zur Pharmakologie von Prächalciferol2 und Lumicalciferol2. *Die Naturwissenschaften*, 50(20), pp.647–648.

Van Berkel, G.J., Zhou, F. & Aronson, J.T., 1997. Changes in bulk solution pH caused by the inherent controlled-current electrolytic process of an electrospray ion source. *International Journal of Mass Spectrometry and Ion Processes*, 162(1–3), pp.55–67.

Bikle, D.D., 2016. Extraskeletal actions of vitamin D. *Annals of the New York Academy of Sciences*, 1376(1), p.29.

Bilodeau, L. et al., 2011. Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. *Journal of Food Composition and Analysis*, 24(3), pp.441–448.

Black, L.J. et al., 2012. An Updated Systematic Review and Meta-Analysis of the Efficacy of Vitamin D Food Fortification. *Journal of Nutrition*, 142(6), pp.1102–1108.

Blomhoff, R. et al., 1984. Lymphatic absorption and transport of retinol and vitami D-3 from rat intestine: Evidence for different pathways. *Biochimica et Biophysica Acta*, 772, pp.109–116.

Bogh, M.K.B. et al., 2011. Vitamin D production depends on ultraviolet-B dose but not on dose rate: A randomized controlled trial. *Experimental Dermatology*, 20(1), pp.14–18.

Bolliger, H.R., 1965. Vitamins. In *Thin-Layer Chromatography*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 210–248.

Bonfiglio, R. et al., 1999. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid communications in mass spectrometry : RCM*, 13(12), pp.1175–1185.

Braun, M. et al., 1991. Improved photosynthesis of previtamin D by wavelengths of 280–300 nm. *Journal of Photochemistry and Photobiology A: Chemistry*, 61(1), pp.15–26.

Browning, L.C. & Cowieson, A.J., 2014. Vitamin D fortification of eggs for human health. *Journal of the science of food and agriculture*, 94(7), pp.1389–96.

Buhrman, D.L., Price, P.I. & Rudewicz, P.J., 1996. Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: A

study of ion suppression. *Journal of the American Society for Mass Spectrometry*, 7(11), pp.1099–1105.
Bunker, J. & Harris, R.S., 1937. Precise evaluation of ultraviolet therapy in experimental rickets. *New England Journal of Medicine*, 216, pp.165–169.
Burild, A. et al., 2014. Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. *Journal of separation science*, 37(19), pp.2659–63.
Burild, A. et al., 2015. Tissue content of vitamin D3 and 25-hydroxy vitamin D3 in minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D3. *Steroids*, 98, pp.72–9.
Burild, A. et al., 2016. Vitamin D3 and 25-hydroxyvitamin D3 in pork and their relationship to vitamin D status in pigs. *Journal of nutritional science*, 5, p.e3.
Byrdwell, W.C. et al., 2008. Analyzing vitamin D in foods and supplements: methodologic challenges. *The American Journal of Clinical Nutrition*, 88(2), p.554S–557S.
Byrdwell, W.C. et al., 2013. Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection and mass spectrometry. *Journal of Food Composition and Analysis*, 30(2), pp.109–119.
Cashman, K.D. et al., 2016. Vitamin D deficiency in Europe: pandemic? *American Journal of Clinical Nutrition*, 103, pp.1033–1044.
Cashman, K.D. & Kiely, M., 2016. Tackling inadequate vitamin D intakes within the population: fortification of dairy products with vitamin D may not be enough. *Endocrine*, 51(1), pp.38–46.
Chen, T.C. et al., 2000. An evaluation of the biologic activity and vitamin D receptor binding affinity of the photoisomers of vitamin D3 and previtamin D3. *The Journal of Nutritional Biochemistry*, 11, pp.267–272.
Christakos, S. et al., 2016. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev*, 96, pp.365–408.
Cisneros, C. et al., 2017. The role of tachysterol in vitamin D photosynthesis – a non-adiabatic molecular dynamics study. *Phys. Chem. Chem. Phys*, 19, p.5763.
Datta, P. et al., 2016. Major inter-personal variation in the increase and maximal level of 25-hydroxy vitamin D induced by UVB. *Photochemical & Photobiological Sciences*, 15(4), pp.536–545.
Dauben, W.G. & Phillips, R.B., 1982. Effects of wavelength on the photochemistry of provitamin D3. *Journal of the American Chemical Society*, 104(21), pp.5780–5781.
DeLuca, H.F., 2004. Overview of general physiologic features and functions of vitamin D. *The American Journal of Clinical Nutrition*, 80(6), p.1689S–1696S.
Van Dijk, A. et al., 2016. The action spectrum for vitamin D 3 : initial skin reaction and prolonged exposure. *Photochemical & Photobiological Sciences*, 15, p.896.
Dimartino, G., 2009. Simultaneous Determination of Cholecalciferol (Vitamin D3) and Ergocalciferol (Vitamin D2) in Foods by Selected Reaction Monitoring. *Journal of AOAC International*, 92(2), pp.511–517.
Ding, S. et al., 2010. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. *Analytical and Bioanalytical Chemistry*, 398(2), pp.779–789.
Dueland, S., Pedersen, J.I., et al., 1983. Absorption, distribution, and transport of vitamin D3 and 25-hydroxyvitamin D3 in the rat. *The American journal of physiology*, 245(5 Pt 1), pp.E463-7.
Dueland, S., Helgerud, P., et al., 1983. Plasma clearance, transfer, and distribution of vitamin D3 from intestinal lymph. *The American journal of physiology*, 245(4), pp.E326-31.

EFSA, 2019. Safety for the environment of vitamin D3 for salmonids. *EFSA Journal*, 17(1). Available at: http://doi.wiley.com/10.2903/j.efsa.2019.5540.
EFSA, 2017. Safety of vitamin D3 addition to feedingstuffs for fish. *EFSA Journal*, 15(3). Available at: http://doi.wiley.com/10.2903/j.efsa.2017.4713.
EFSA, 2014. Scientific Opinion on the safety and efficacy of vitamin D3 (cholecalciferol) as a feed additive for all animal species or categories based on a dossier submitted by Lohmann Animal Health GmbH. *EFSA Journal*, 12(2), p.3568.
EFSA NDA Panel, 2014. EFSA Panel on Dietetic Products, Nutrition and Allergies. Scientific Opinion on the safety of vitamin D-enriched UV-treated baker's yeast. EFSA Journal 2014;12(1):3520, 19 pp.
Engelsen, O., 2010. The relationship between ultraviolet radiation exposure and vitamin D status. *Nutrients*, 2(5), pp.482–95.
Furey, A. et al., 2013. Ion supression; A critical review on causes , evaluation , prevention and applications. *Talanta*, 115, pp.104–122.
Gill, B.D. et al., 2016. Analysis of Vitamin D 2 and Vitamin D3 in Fortified Milk Powders and Infant and Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry: Single-Laboratory Validation, First Action 2016.05. *Journal of AOAC International*, 99(5), pp.1321–1330.
Gill, B.D. et al., 2019. Significance of Thermal Isomerisation on the Quantitation of Total Vitamin D3 in Foods. *Food Analytical Methods*, (Online first), pp.1–9.
Gilliland, D.L. et al., 2012. Simultaneous Determination of Vitamins D 2 and D 3 by LC-MS/MS in Infant Formula and Adult Nutritionals: First Action 2011.13. *Journal of AOAC International*, 95(3), pp.583–587.
Gomes, F.P. et al., 2015. Simultaneous quantitative analysis of eight vitamin D analogues in milk using liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta*, 891, pp.211–220.
Grigalavicius, M. et al., 2015. Vitamin D and ultraviolet phototherapy in Caucasians. *Journal of Photochemistry and Photobiology B: Biology*, 147, pp.69–74.
Hanewald, K.H., Mulder, F.J. & Keuning, K.J., 1968. Thin layer chromatographic assay of vitamin D in high potency preparations - analysis of fat soluble vitamins IX. *Journal of Pharmaceutical Sciences*, 57(8), pp.1308–1312.
Hanewald, K.H., Rappoldt, M.P. & Roborgh, J.R., 1961. The antirachitic activity of previtamin D3. *Recueil des Travaux Chimiques des Pays-Bas*, 80(9), pp.1003–1014.
Havinga, E., 1960. the Photochemical Interconversions Provitamin Previtamin and Tachysterol of. *Tetrahedron*, 11, pp.276–284.
Heaney, R.P., 2014. Toward a physiological referent for the vitamin D requirement. *J Endocrinol Invest*, 37, pp.1127–1130.
Heaney, R.P. et al., 2009. Vitamin D 3 Distribution and Status in the Body Vitamin D 3 Distribution and Status in the Body. *Journal of the American College of Nutrition*, 28(3), pp.252–256.
Heudi, O., Trisconi, M.-J. & Blake, C.-J., 2004. Simultaneous quantification of Vitamins A, D3 and E in fortified infant formulae by liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 1022, pp.115–123.
Higashi, T. et al., 2008. Liquid chromatography-tandem mass spectrometric method for the determination of salivary 25-hydroxyvitamin D3: a noninvasive tool for the assessment of vitamin D status. *Analytical and bioanalytical chemistry*, 391(1), pp.229–38.
Higashi, T., Shimada, K. & Toyo'oka, T., 2010. Advances in determination of vitamin D related compounds in biological samples using liquid chromatography-mass spectrometry: a review. *Journal of chromatography. B, Analytical technologies in*

the biomedical and life sciences, 878(20), pp.1654–61.
Holick, M.F. et al., 1995. Evolutionary importance for the membrane enhancement of the production of vitamin D3 in the skin of poikilothermic animals. *Proceedings of the National Academy of Sciences of the United States of America*, 92(8), pp.3124–3126.
Holick, M.F. et al., 1979. Isolation and identification of previtamin D3 from the skin of rats exposed to ultraviolet irradiation. *Biochemistry*, 18(6), pp.1003–1008.
Holick, M.F. et al., 1980. Photosynthesis of Previtamin D3 in Human Skin And the Physiologic Consequences. *Science*, 210(4466), pp.203–205.
Holick, M.F. & Hossein-Nezhad, A., 2017. The D-lemma: narrow-band UV type B radiation versus vitamin D supplementation versus sunlight for cardiovascular and immune health. *American Journal of Clinical Nutrition*.
Holick, M.F., MacLaughlin, J.A. & Doppelt, S.H., 1981. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. *Science*, 211(4482), pp.590–593.
Höller, U. et al., 2010. Rapid determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay. *Journal of Chromatography B*, 878(13–14), pp.963–968.
Housecroft, C.E. & Constable, E.C., 2006. *Chemistry : an introduction to organic, inorganic, and physical chemistry* third edit., Pearson Prentice Halll.
Huang, M. et al., 2012. Application of Ultra-High-Performance Liquid Chromatography/ Tandem Mass Spectrometry for the Measurement of Vitamin D in Infant Formula and Adult/Pediatric Nutritional Formula: First Action 2011.11. *Journal of AOAC International*, 95(2), pp.319–321.
Huang, M., Cadwallader, A.B. & Heltsley, R., 2014. Mechanism of error caused by isotope-labeled internal standard: accurate method for simultaneous measurement of vitamin D and pre-vitamin D by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, 28(19), pp.2101–2110.
Institute of Medicine, 1997. *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride*, Washington DC: National Academies Press.
Institute of Medicine, 2011. *Dietary Reference Intakes for Vitamin D and Calcium*, Washington DC: The National Academies Press.
International Commission on Illumination, 1999. Erythema Reference Action Spectrum and Standard Erythema Dose. ISO/CIE Standard ISO 17166:1999.
Jakobsen, J. et al., 2007. 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin D. *British Journal of Nutrition*, 98(05), pp.908–913.
Jakobsen, J., 2007. Bioavailability and bioactivity of vitamin D3 active compounds - Which potency should be used for 25-hydroxyvitamin D3 ? *International Congress Series*, 1297, pp.133–142.
Jakobsen, J. et al., 2015. Short communication: Artificial ultraviolet B light exposure increases vitamin D levels in cow plasma and milk. *Journal of dairy science*, pp.6492–6498.
Jakobsen, J. et al., 2018. Vitamin D Vitamers Affect Vitamin D Status Differently in Young Healthy Males. *Nutrients*, 10(1), p.12.
Jäpelt, R.B. et al., 2013. Quantification of vitamin D3 and its hydroxylated metabolites in waxy leaf nightshade (Solanum glaucophyllum Desf.), tomato (Solanum lycopersicum L.) and bell pepper (Capsicum annuum L.). *Food Chemistry*, 138(2–3), pp.1206–1211.
Jones, G., 2008. Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr*, 88, p.582S–586S.

Kawazoe, T. et al., 1994. Production of Vitamin D-fortified eggs by feeding vitamin D2-fortified shiitake. *Nippon Shokuhin Kagaku Kogaku Kaishi*, 41(12), pp.891–896.
Keegan, R.J.H. et al., 2013. Photobiology of vitamin D in mushrooms and its bioavailability in humans. *Dermato-Endocrinology*, 5(1), pp.165–176.
Khan, M.A. et al., 2019. Improved external quantum efficiency of 293 nm AlGaN UVB LED grown on an AlN template. *Japanese Journal of Applied Physics*, 58(SA), p.SAAF01.
Kiely, M. & Black, L.J., 2012. Dietary strategies to maintain adequacy of circulating 25-Hydroxyvitamin D concentrations. *Scandinavian Journal of Clinical and Laboratory*, 72, pp.14–23.
Knudson, A. & Benford, F., 1938. Ultraviolet Radiation in Rickets. *Journal of Biological Chemistry*, 124, pp.287–299.
Kobayashi, T., Hirooka, M. & Yasumura, M., 1976. Effect of wavelength on the ultraviolet irradiation of 7-dehydrocholesterol. *Vitamins (in Japanese)*, 50, pp.185–189.
Kobayashi, T. & Yasumura, M., 1973. Studies on the Ultraviolet Irradiation of Provitamin D and Its Related Compounds Iii. Effect of Wavelength on the for-Mation of Potential Vitamin D2 in the Irradiation of Ergosterol By Mono-Chromatic Ultraviolet Rays. *Journal of Nutritional Science and Vitaminology*, 19(2), pp.123–128.
Kolp, E. et al., 2017. Vitamin D metabolism in growing pigs: influence of UVB irradiation and dietary vitamin D supply on calcium homeostasis, its regulation and bone metabolism. *Journal of Animal Physiology and Animal Nutrition*, 101(Suppl. 1, Sp. Iss. SI), pp.79–94.
Konermann, L., 2017. Addressing a Common Misconception: Ammonium Acetate as Neutral pH “Buffer” for Native Electrospray Mass Spectrometry. *Journal of The American Society for Mass Spectrometry*, 28(9), pp.1827–1835.
Konermann, L. et al., 2013. Unraveling the mechanism of electrospray ionization. *Analytical Chemistry*, 85(1), pp.2–9.
Kostiainen, R. & Kauppila, T.J., 2009. Effect of eluent on the ionization process in liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 1216, pp.685–699.
Kristensen, H.L., Rosenqvist, E. & Jakobsen, J., 2012. Increase of vitamin D2 by UV-B exposure during the growth phase of white button mushroom (Agaricus bisporus). *Food & nutrition research*, 56, pp.1–7.
Kühn, J. et al., 2014. Free-range farming: A natural alternative to produce vitamin D-enriched eggs. *Nutrition*, 30(4), pp.481–484.
Kühn, J. et al., 2015. Non-linear increase of vitamin D content in eggs from chicks treated with increasing exposure times of ultraviolet light. *The Journal of Steroid Biochemistry and Molecular Biology*, 148, pp.7–13.
Larson-Meyer, D.E. et al., 2017. Sun exposure in pigs increases the Vitamin D nutritional quality of pork. *PLoS ONE*, 12(11).
Lawson, D.E. et al., 1979. Relative contributions of diet and sunlight to vitamin D state in the elderly. *British medical journal*, 2(6185), pp.303–305.
Lehmann, B. et al., 2001. UVB-induced conversion of 7-dehydrocholesterol to 1α,25-dihydroxyvitamin D3 in an in vitro human skin equivalent model. *The Journal of investigative dermatology*, 117(5), pp.1179–1185.
Lehmann, B., Knuschke, P. & Meurer, M., 2007. The UVB-induced synthesis of vitamin D3 and 1α,25-dihydroxyvitamin D3 (calcitriol) in organotypic cultures of keratinocytes: Effectiveness of the narrowband Philips TL-01 lamp (311 nm). *Journal of Steroid Biochemistry and Molecular Biology*, 103(3–5), pp.682–685.
Lietzow, J. et al., 2012. Effect of short-term UVB exposure on vitamin D concentration

of eggs and vitamin D status of laying hens. *Journal of agricultural and food chemistry*, 60(3), pp.799–804.
Lipkie, T.E. et al., 2013. Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, 932, pp.6–11.
Luce, E.M., 1924. CLXVIII . Further observations on the influence of sunlight upon the growth-promoting and anti-rachitic properties of cow's milk. *The Biochemical journal*, 18, pp.1279–1288.
MacLaughlin, J.A., Anderson, R.R. & Holick, M.F., 1982. Spectral character of sunlight modulates photosynthesis of previtamin D3 and its photoisomers in human skin. *Science*, 216(4549), pp.1001–1003.
Mahmoodani, F. et al., 2017. Degradation studies of cholecalciferol (vitamin D3) using HPLC-DAD, UHPLC-MS/MS and chemical derivatization. *Food Chemistry*, 219, pp.373–381.
Mallet, C.R., Lu, Z. & Mazzeo, J.R., 2004. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry*, 18(1), pp.49–58.
Mattila, P. et al., 2003. Effect of cholecalciferol-enriched hen feed on egg quality. *Journal of agricultural and food chemistry*, 51(1), pp.283–7.
Mattila, P. et al., 2004. Effect of vitamin D2- and D3-enriched diets on egg vitamin D content, production, and bird condition during an entire production period. *Poultry science*, 83(3), pp.433–40.
Mattila, P., Valkonen, E. & Valaja, J., 2011. Effect of different vitamin D supplementations in poultry feed on vitamin D content of eggs and chicken meat. *Journal of agricultural and food chemistry*, 59(15), pp.8298–303.
Matuszewski, B.K., 2006. Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. *Journal of Chromatography B*, 830(2), pp.293–300.
Matuszewski, B.K., Constanzer, M.L. & Chavez-Eng, C.M., 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, 75(13), pp.3019–3030.
Maughan, G., 1928. Ultra-violet wave lengths valuable in the cure of rickets in chickens. *American Journal of Physiology*, 87(2), pp.381–398.
Nordic Council of Ministers, 2012. Nordic Nutrition Recommendations 2012 : Integrating nutrition and physical activity.
Norval, M., Björn, L.O. & de Gruijl, F.R., 2010. Is the action spectrum for the UV-induced production of previtamin D3 in human skin correct? *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology*, 9(1), pp.11–17.
O'Brien, B., McEwen, H. & Morgareidge, K., 1938. Irradiated milk: Vitamin D potency as a function of energy input. *Industrial & Engineering Chemistry*, 30(7), pp.839–842.
O'Mahony, L. et al., 2011. The potential role of vitamin D enhanced foods in improving vitamin D status. *Nutrients*, 3(12), pp.1023–41.
O'Neill, C. et al., 2016. Seasonal Changes in Vitamin D-Effective UVB Availability in Europe and Associations with Population Serum 25-Hydroxyvitamin D. *Nutrients*, 8(9), p.533.
Oh, S.-T. et al., 2019. Designing Safe General LED Lighting that Provides the UVB Benefits of Sunlight. *Applied Sciences*, 9(5), p.826.
Okano, T. et al., 1978. In vivo and in vitro conversion of 7-dehydrocholesterol into vitamin D3 in rat skin by ultraviolet ray's irradiation. *Journal of nutritional science and vitaminology*, 24(1), pp.47–56.

Olds, W.J., Lucas, R.M. & Kimlin, M.G., 2010. Action Spectrum for Vitamin D Synthesis. *UV Radiation and its effects - 2010, NIWA UV workshop. 7-9 April 2010. NIWA Information Series No. 77. Copthorne Hotel, Queenstown, New Zealand*, pp.106–107.

Ovesen, L., Brot, C. & Jakobsen, J., 2003. Food contents and biological activity of 25-hydroxyvitamin D: A vitamin D metabolite to be reckoned with? *Annals of Nutrition and Metabolism*, 47(3–4), pp.107–113.

Persia, M.E. et al., 2013. Effects of long-term supplementation of laying hens with high concentrations of cholecalciferol on performance and egg quality. *Poultry science*, 92(11), pp.2930–7.

Pilz, S. et al., 2018. Rationale and Plan for Vitamin D Food Fortification: A Review and Guidance Paper. *Frontiers in Endocrinology*, 9, p.373.

Pilz, S. et al., 2016. Vitamin D and cardiovascular disease prevention. *Nature Reviews Cardiology*, 13(7), pp.404–417.

Poskitt, E.M., Cole, T.J. & Lawson, D.E., 1979. Diet, sunlight, and 25-hydroxy vitamin D in healthy children and adults. *British medical journal*, 1(6158), pp.221–223.

Quesada-Gomez, J.M. & Bouillon, R., 2018. Is calcifediol better than cholecalciferol for vitamin D supplementation? *Osteoporosis International*, 29(8), pp.1697–1711.

Reboul, E., 2015. Intestinal absorption of vitamin D: from the meal to the enterocyte. *Food & Function*, 6, pp.356–362.

Rejnmark, L. et al., 2017. Non-skeletal health effects of Vitamin D supplementation: A systematic review on findings from meta-Analyses summarizing trial data. *PLoS ONE*, 12(7).

Schadt, H.S. et al., 2012. Quantification of Vitamin D3 in Feed, Food, and Pharmaceuticals Using High-Performance Liquid Chromatography/Tandem Mass Spectrometry. *Journal of AOAC International*, 95(5), pp.1487–1494.

Schutkowski, A. et al., 2013. UVB exposure of farm animals: study on a food-based strategy to bridge the gap between current vitamin D intakes and dietary targets. *PloS one*, 8(7), p.e69418.

Shaw, W.H.C. & Jefferies, J.P., 1957. The determination of vitamin D and related compounds. Part II. Analysis of irradiation products. *The Analyst*, 82(970), p.8.

Slominski, A.T. et al., 2017. Characterization of a new pathway that activates lumisterol in vivo to biologically active hydroxylumisterols. *Scientific Reports*, 7(1).

Souza, C.D. et al., 2015. Application of Light-Emitting Diodes in Food Production, Postharvest Preservation, and Microbiological Food Safety. *Comprehensive Reviews in Food Science and Food Safety*, 14, pp.719–740.

de Souza, P.M. & Fernández, A., 2013. Rheological properties and protein quality of UV-C processed liquid egg products. *Food Hydrocolloids*, 31(1), pp.127–134.

Standing Committee on Plant, Animals, F. and F.S.A.N., 2019. *Standing Committee on Plants, Animals, Food and Feed Section Animal Nutrition. 09-11 April 2019. Agenda*, Available at: https://ec.europa.eu/food/animals/committees/sc_animal-nutrition_en [Accessed April 14, 2019].

Steenbock, H., Hart, E.B. & Jones, J.H., 1924. Fat-soluble vitamins. XVIII. Sunlight in its relation to pork production on certain restricted rations. *Journal of Biological Chemistry*, 61, pp.775–794.

Stevens, J. & Dowell, D., 2012. Determination of Vitamins D2 and D3 in Infant Formula and Adult Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection (UPLC-MS/MS): First Action 2011.12. *Journal of AOAC International*, 95(3), pp.577–582.

Strobel, N. et al., 2013. Vitamin D and 25-hydroxyvitamin D determination in meats by LC–IT-MS. *Food Chemistry*, 138(2–3), pp.1042–1047.

Takada, K. et al., 1979. A rapid and precise method for the determination of vitamin D3

in rat skin by high-performance liquid chromatography. *Journal of nutritional science and vitaminology*, 25(5), pp.385–398.
Terry, M. et al., 1999. Safety of 25-hydroxyvitamin D3 as a source of vitamin D3 in layer poultry feed. *Veterinary and human toxicology*, 41(5), pp.312–6.
The Danish Environmental Protection Agency, Optimal lighting-system for pigs / PigLED. Available at: https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2016-projekter/optimal-lighting-system-for-pigs-pigled/ [Accessed April 30, 2019].
Tian, X.Q. et al., 1994. Characterization of the translocation process of vitamin D3 from the skin into the circulation. *Endocrinology*, 135(2), pp.655–661.
Tian, X.Q. et al., 1993. Kinetic and thermodynamic studies of the conversion of previtamin D3 to vitamin D3 in human skin. *Journal of Biological Chemistry*, 268(20), pp.14888–14892.
Trenerry, V.G. et al., 2011. The determination of vitamin D3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemistry*, 125(4), pp.1314–1319.
Trufelli, H. et al., 2011. An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*, 30(3), pp.491–509.
Underwood, J.L. & DeLuca, H.F., 1984. Vitamin D is not directly necessary for bone growth and mineralization. *Am J Physiol*, 246(6 Pt 1), pp.E493-8.
Urbain, P. & Jakobsen, J., 2015. Dose−Response Effect of Sunlight on Vitamin D 2 Production in Agaricus bisporus Mushrooms. *J. Agric. Food Chem*, 63, p.8161.
Uva, B., Mandich, A. & Vallarino, M., 1983. The site of 7-dehydrocholesterol ultraviolet photolysis in domestic fowls. *Acta Histochemica*, 73(2), pp.175–180.
Wang, S., Cyronak, M. & Yang, E., 2007. Does a stable isotopically labeled internal standard always correct analyte response?. A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, 43(2), pp.701–707.
Webb, A.R., 2006. Who, what, where and when-influences on cutaneous vitamin D synthesis. *Progress in Biophysics and Molecular Biology*, 92(1), pp.17–25.
Webb, A.R., DeCosta, B.R. & Holick, M.F., 1989. Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. *Journal of Clinical Endocrinology and Metabolism*, 68(5), pp.882–887.
Webb, A.R., Kline, L. & Holick, M.F., 1988. Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. *The Journal of clinical endocrinology and metabolism*, 67(2), pp.373–8.
Wieling, J., 2006. LC-MS-MS experiences with internal standards. *Chromatographia*, 55(S1), pp.S107–S113.
Woollard, D.C., Indyk, H.E. & Gill, B.D., 2019. Significance of previtamin D chromatographic resolution in the accurate determination of vitamin D3 by HPLC–UV. *Journal of Food Composition and Analysis*, 79, pp.1–4.
Wu, J. et al., 2013. A stable isotope-labeled internal standard is essential for correcting for the interindividual variability in the recovery of lapatinib from cancer patient plasma in quantitative LC-MS/MS analysis. *Journal of Chromatography B*, 941, pp.100–108.
Yamamoto, J.K. & Borch, R.F., 1985. Photoconversion of 7-Dehydrocholesterol to Vitamin D3 in Synthetic Phospholipid Bilayers. *Biochemistry*, 24, pp.3338–3344.
Yang, J., Cleland, G. & Organtini, K., 2017. *Determination of Vitamin D and previtamin D in Food Products*, Milford.

Publications

Paper I: **Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin**
Line Lundbæk Barnkob, Aikaterini Argyraki, Paul Michael Petersen and Jette Jakobsen
Food Chemistry, 2016; 212; 386-391.

Paper II: **Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities**
Line Lundbæk Barnkob, Paul Michael Petersen, Jens Peter Nielsen and Jette Jakobsen
European Food Research and Technology, 2018; 245(2); 311-418.

Paper III: **Naturally enhanced eggs as a source of vitamin D: A review**
Line Lundbæk Barnkob, Aikaterini Argyraki and Jette Jakobsen
Submitted to Trends in Food Science and Technology

Paper IV: **Deuterated internal standard in the analysis of vitamin D in foods does not always correct for matrix effects: the effect of eluent additives**
Line Lundbæk Barnkob, Henrik Lauritz Frandsen and Jette Jakobsen
Prepared for submission to Journal of Chromatography A

Paper I

Food Chemistry 212 (2016) 386–391

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin

Line Lundbæk Barnkob [a], Aikaterini Argyraki [b], Paul Michael Petersen [b], Jette Jakobsen [a,*]

[a] National Food Institute, Technical University of Denmark, Denmark
[b] Department of Photonics Engineering, Technical University of Denmark, Denmark

ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 8 May 2016
Accepted 24 May 2016
Available online 24 May 2016

Keywords:
Vitamin D
Bio-fortification
Light-emitting diodes
Ultra violet light

ABSTRACT

The dietary intake of vitamin D is currently below the recommended intake of 10–20 μg vitamin D/day. Foods with increased content of vitamin D or new products with enhanced vitamin D are warranted. Light-emitting diodes (LEDs) are a potential new resource in food production lines. In the present study the exposure conditions with ultraviolet (UV) LEDs were systematically investigated in the wavelength range 280–340 nm for achieving optimal vitamin D bio-fortification in pig skin. A wavelength of 296 nm was found to be optimal for vitamin D_3 production. The maximum dose of 20 kJ/m^2 produced 3.5–4 μg vitamin D_3/cm^2 pig skin. Vitamin D_3 produced was independent on the combination of time and intensity of the LED source. The increased UV exposure by UV-LEDs may be readily implemented in existing food production facilities, without major modifications to the process or processing equipment, for bio-fortifying food products containing pork skin.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

The recommended human dietary intake of vitamin D is in the range 10–20 μg/day (Institute of Medicine, 2011; Nordic Nutrient Recommendations, 2014). However typical recorded dietary intakes are insufficient. Dietary supplements could be used to close the gap, however, it is not an appropriate strategy to increase intakes across the population, because uptake does not typically exceed 40%. An effective food-based strategy could increase dietary intake among the population (Black, Seamans, Cashman, & Kiely, 2012; O'Mahony, Stepien, Gibney, Nugent, & Brennan, 2011). The optimal procedure seems to be either the fortification of a broad range of foods, or to increase the content of vitamin D in foods that are already sources of vitamin D.

The natural content of vitamin D in our foods varies widely. Cod liver oil contains 250 μg vitamin D/100 g; fatty fish such as salmon, eel and mackerel contain 8–30 μg/100 g, lean fish such as halibut, sole and tuna contain 3–9 μg/100 g; while meat and dairy products contain less than 1 μg/100 g (Saxholt et al., 2008). However, due to the high dietary intake of the latter food products, the contribution of vitamin D from meat and dairy products is essential, especially in populations with limited availability of fortified food (Pedersen et al., 2015).

Fortification by adding vitamin D to the final product (e.g. milk, margarine and bread) has been introduced in some countries. However, another strategy is bio-fortification by adding more vitamin D to the feed of production animals. However, there are maximum limits for the addition of vitamin D in feed in Europe (EEC, 2004) which reduce the potential advantage of bio-fortifying through feed. In Denmark, the feed for laying hens contains the maximum allowed dose, 3000 IU vitamin D/kg feed. For pigs, there is potential to approximately double the vitamin D content in the meat as the current recommendation of 800 IU vitamin D/kg feed is below the maximum allowed 2000 IU vitamin D/kg (Burild, Lauridsen, Faqir, Sommmer, & Jakobsen, 2016).

It is possible to convert 7-dehydrocholesterol (7-DHC) to vitamin D_3 by exposing excised skin to ultraviolet B (UVB, 290–320 nm) light (MacLaughlin, Anderson, & Holick, 1982; Okano, Yasumura, Mizuno, & Kobayashi, 1978). When 7-DHC, which is located in the two outer layers of the skin (the epidermis and dermis located on top of the subcutaneous fat), is exposed to UVB light it is transformed to pre-vitamin D_3 (preD_3), which isomerises to form vitamin D_3 (Holick et al., 1980). Therefore, an alternative bio-fortification strategy is to expose animals or foodstuff, containing 7-DHC or ergosterol to UV light to increase the level of vitamin D_3 and vitamin D_2, respectively. The principle is approved for use in the production of vitamin D_2 – enriched baker's yeast in the United States (Food & Drug Administration, 2012) and in the European Union (EFSA NDA Panel, 2014). Exposure of

* Corresponding author.
E-mail address: jeja@food.dtu.dk (J. Jakobsen).

http://dx.doi.org/10.1016/j.foodchem.2016.05.155
0308-8146/© 2016 Elsevier Ltd. All rights reserved.

mushrooms to UVB light has been shown to increase vitamin D content in a wavelength-dependent manner (Jasinghe & Perera, 2006). Recent studies have shown that UVB-exposure of dairy cows and pigs can enhance the content of vitamin D in milk and pork (Burild, Frandsen, Poulsen, & Jakobsen, 2015; Jakobsen et al., 2015).

To use this principle in the production of food requires the construction of a light-source that can accommodate all the requirements existing in food production lines. With the emerging technology of ultraviolet light-emitting diodes (UV-LEDs) it is possible to produce energy-efficient UV light sources with a narrow and tuned UVB-spectrum, which could ensure an environmentally friendly, cost-effective production process.

Thus we aimed to study the feasibility of using UV-LEDs in the production of vitamin D – enhanced pork products. We investigated how the exposure conditions, namely: wavelength, dose and total irradiation and exposure time, can influence vitamin D_3 production when pig skin without hair is exposed to UV light, produced by UV-LEDs.

2. Materials and methods

2.1. Samples of pork skin

Skin was removed from the back of a slaughtered mini pig which had been in the control group of one of our former studies (Burild et al., 2015). Thus had never been exposed to UV light, but stored at −20 °C for 2 years prior to the removal of the skin. Any hair and subcutaneous fat was carefully removed from the skin. A normal ruler and a scalpel were used to cut the skin into pieces of 1 × 1 cm. The average weight of the samples was 0.498 ± 0.015 g. All samples were kept at −20 °C before and after exposure to UV light. Prior to exposure the samples were thawed to room temperature. After exposure the samples were kept in airtight nitrogen-flushed bags. Control-samples, i.e. skin samples which were not exposed to UV light, were included in the study. All samples before and after the experiment were kept in an UV-free environment.

2.2. The UV-LED equipment

Twelve UV-LEDs, emitting wavelengths in the range 280–340 nm, were purchased from Sensor Electronic Technology, Inc (SETi, Columbia, SC, USA; TO3 package, hemispherical lens window, half angle of 20–25°). An UV opaque, homemade Plexiglass (RIAS A/S, Roskilde, Denmark) box was built and used to protect the experimentalist against the UV light. The irradiation of the UV-LEDs was measured by an External Optical probe (EOP-146, Instrument Systems GmbH, Munich, Germany) and a monochromator (bandpass: 1 nm, scan step: 1 nm, detector: Photomultiplier). The spectrometer, coupled to the monochromator, was a SPECTRO 320 (D) Release 5 (Instrument Systems GmbH) and operated in the wavelengths between 200 nm and 900 nm. The spectral distribution for each of the twelve LEDs was systematically investigated in six constant current modes: 100 mA, 200 mA, 300 mA, 400 mA, 500 mA and 600 mA. The measurements were performed with contact between the detector and the light source, and afterwards a correction was introduced for the distance introduced between the sample and light source. The relation between total irradiation and distance was measured and is displayed in Fig. 1. Gaussian curves were fitted to the six spectral distributions obtained for each of the LEDs. Based on the Gaussian fit the central wavelength, standard deviation and full width half maximum (FWHM) were estimated. The range was determined as plus/minus three standard deviations. Total irradiation was calculated by taking the integral of irradiations of all emitted wavelengths for each LED. The estimated values are displayed in Table 1.

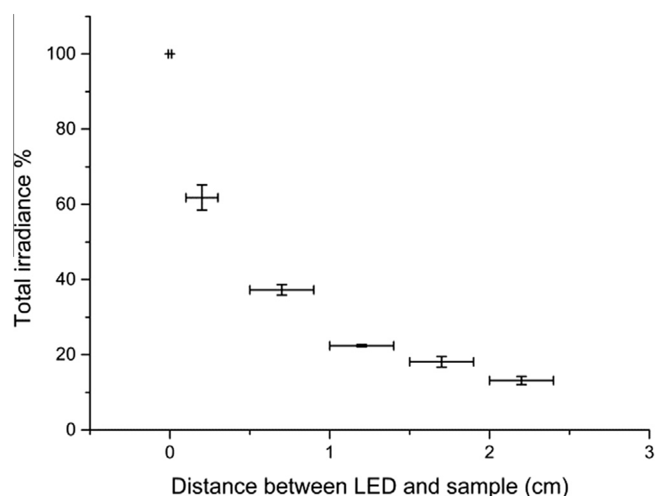


Fig. 1. Relation between total irradiation percentage delivered on the sample and distance between LED and sample at a constant current of 600 mA.

Table 1
The central wavelength, full width at half maximum (FWHM).

Central wavelength (nm)		FWHM (nm)	Range[a] (nm)
Purchased as	Measured as		
280	281	11	266–296
285	285	11	270–300
290	292	10	280–304
295	296	9	284–308
300	300	10	288–312
305	306	11	291–321
310	310	10	298–322
315	313	10	301–325
320	318	10	306–330
330	330	16	309–351
335	336	12	321–351
340	338	11	323–353

[a] Range (±3 sd) were estimated based on the Gaussian fit of the spectral distribution.

2.3. Experimental design

All provided values are given as mean ± standard deviation (sd).

The objective was to determine how wavelength, dose and total irradiation influence vitamin D_3 production when pig skin is exposed to UV. In all exposures, the distance between the LED's and the sample of pig skin (1 cm^2) was kept constant at 1.5 ± 0.1 cm. All exposures were repeated on two samples of pig skin (n = 2). Pictures of the setup and the exact settings used for the LEDs in each experiment can be found in the Supplementary online material (SOM, Section S1, Fig. S1).

2.3.1. Effect of wavelength on the production of vitamin D_3 in pig skin

The effect of wavelength on the production of vitamin D_3 was tested at two different doses; namely 300 ± 3 J/m^2 and 7,000 ± 3 J/m^2. The total irradiation emitted at these wavelengths varied between 8.2–12.4 W/m^2 and 2–23 W/m^2, respectively. The corresponding range in exposure time was 24–37 sec and 304–3500 s, respectively. The precision of the exposure time was estimated to ±0.5 s to cover the experimentalist's response time to the timer. The LEDs were operated in constant current mode in the region between 100 and 600 mA (see SOM, Section S2, Table S1).

2.3.2. Effect of dose on the production of vitamin D_3 in pig skin

The LED, with a central wavelength of 296 nm, was used to determine the effect of 6 doses on the vitamin D_3 production in pig skin. The delivered doses were 207 J/m^2, 1008 J/m^2, 2001 J/m^2, 6002 J/m^2, 10,004 J/m^2 and 20,007 J/m^2. The exposure time was varied between 14 s and 22.50 min (see SOM, Section S2, Table S2). The LED was operated at a constant current of 600 mA and the total irradiation emitted was 14.8 W/m^2.

2.3.3. Effect of total irradiation and exposure time on the production of vitamin D_3 in pig skin

The total irradiation emitted by the LEDs was varied between 0.1 and 43 W/m^2 by adjusting the operation current at the interval from 8 to 600 mA. The test was performed at three different central wavelengths: 292 nm, 296 nm, and 300 nm and delivered a constant dose of 300 ± 2 J/m^2 (see SOM, Section S2, Table S3).

2.4. Analysis of vitamin D_3 and 7-DHC

2.4.1. Chemicals

The standards used were vitamin D_3 and vitamin D_2 from Sigma-Aldrich (Denmark A/S, Copenhagen, DK), and 7-DHC from Cayman Chemical (Ann Arbor, MI, USA). Concentrations of the standard solutions were determined spectrophotometrically, based on the molar absorption coefficient at 265 nm for vitamin D_3 and D_2 assessed as 18,300 M^{-1} cm^{-1} and 19,400 M^{-1} cm^{-1}, respectively (Norman, 1979) and for 7-DHC at 281 nm: 11,959 M^{-1} cm^{-1}. The value for 7-DHC was obtained from the designated vitamin D_2 equivalent, ergosterol (Sternberg, Stillo, & Schwendeman, 1960).

2.4.2. Procedure

The content of vitamin D_3 and 7-DHC were quantified by combining two methods formerly used for the quantification of vitamin D in meat and mushrooms (Burild, Frandsen, Poulsen, & Jakobsen, 2014; Kristensen, Rosenqvist, & Jakobsen, 2012). In short, the skin samples of 1 cm^2 were thawed prior to analysis, and vitamin D_2 was added. The samples were extracted by alkaline saponification overnight at room temperature and cleaned up using liquid-liquid-extraction followed by silica solid-phase-extraction (Burild et al., 2014), followed by normal-phase preparative HPLC (Kristensen et al., 2012). Vitamin D_2 and vitamin D_3 had a retention time of 7.6 min, and the fraction in the interval 6.8–8.5 min was collected for all samples. The 7-DHC fraction with the retention time of 10 min was collected in fractions 9.2–10.6, but only in the unexposed pig skin. Following evaporation and dissolution in the mobile phase (acetonitrile:methanol, 80:20), an isocratic separation of vitamin D_2 and vitamin D_3 was performed on two C18 columns (VYDAC® 201TP, 5 µm, 250 × 4.6 mm, Separation Group, Inc., Hesperia, CA, USA); whereas the fraction of 7-DHC was separated on a C18 column (Luna®, 5 µm, 250x4.6 mm, Phenomenex, Torrance, CA, USA). A photo-diode array detector (220–320 nm) was used for detection, and quantification at 265 nm for vitamin D_2 and vitamin D_3, and 281 nm for 7-DHC. Vitamin D_2 was used as internal standard for vitamin D_3, whereas 7-DHC was quantified by use of the external standard. The recovery of vitamin D_3 and 7-DHC were >90%. For vitamin D_3, the limit of quantification (LOQ) was 0.003 µg/cm^2 pig skin (equals 0.6 µg/100 g pig skin), and an internal reproducibility at 5.5% in a house reference materials of salmon analysed in each series (n = 8). The analyses were performed in a laboratory accredited according to ISO17025 (ISO, 2005).

3. Results and discussion

3.1. Production of vitamin D_3 as a function of wavelength

The content of vitamin D_3 in the pig skin after a UV dose of 300 J/m^2 and 7000 J/m^2 was delivered, is displayed in Fig. 2 as a function of wavelength. The curves for both doses have a similar shape, with a maximum at 296 nm, but differing in the maximum vitamin D_3 content. Negligible amounts of vitamin D3 were produced at 318 nm, while no vitamin D_3 production was observed at or above 330 nm. At these wavelengths the exposed samples did not differ in content of vitamin D from the unexposed samples i.e. the vitamin D content was below LOQ.

The curves of vitamin D against wavelength (usually described as mountain shaped) are also observed when human skin, rat skin, and 7-DHC and ergosterol solutions are exposed to UV light (Kobayashi & Yasumura, 1973; MacLaughlin et al., 1982; Olds, Lucas, & Kimlin, 2010; Takada, Okano, Tamura, Matsui, & Kobayashi, 1979). Estimation of optimal wavelengths has been assessed under a range of test conditions which have been summarised in Table 2.

All results, no matter the method, assess the optimal wavelength to be in the range 295–303 nm. Furthermore, the production is very low or non-existing above 310 nm. All this is in accordance with our findings. The novelty in our study is that we used UV-LEDs to create narrowband UV light, whereas all others have used traditional UV-sources coupled to either monochromators or filters.

3.2. Production of vitamin D_3 as a function of dose

At the optimum wavelength, 296 nm, the content of vitamin D_3 was determined at six different doses, and is displayed in Fig. 3. The best fitted curve was a logarithmic curve (y = 0.6302 LN(x) – 2.9049) showing a correlation coefficient (R^2) at 0.86.

In human skin, the outer part of epidermis contains a limited amount of 7-DHC, which is mainly present in the deepest layer of epidermis (stratum spinosum and stratum basale), although the deeper layer, the dermis, has also been shown to contain

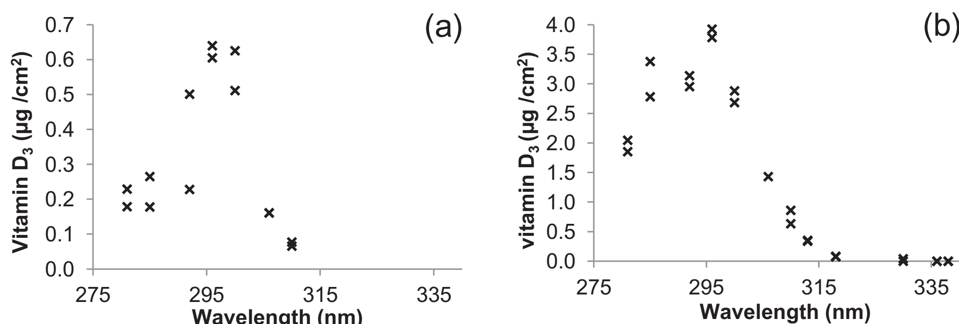


Fig. 2. Content of vitamin D_3 in pig skin as function of wavelength after a delivered dose of a) 300 J/m^2 (281–310 nm) and b) 7000 J/m^2 (281–336 nm).

Table 2
References for investigation of optimum and no production of vitamin D. Information on sample type and full with half maximum (FWHM).

Sample type	Optimum (nm)	No production (nm)	FWHM (nm)	References (year)
Rachitic chickens	296.7	313	No info	Maughan (1928)
Rachitic rats	296.7	313	No info	Bunker and Harris (1937)
Rachitic rats	280.4–302.4	No info	No info	Knudson and Benford (1938)
Ergosterol	295	340	No info	Kobayashi and Yasumura (1973)
Rat skin	303	340	No info	Takada et al. (1979)
7-Dehydrocholesterol	295	No info	No info	Kobayashi, Hirooka, and Yasumura (1976)
Human skin	297	>320	3 or 5	MacLaughlin et al. (1982)
In vitro human skin models	302	315	5	Lehmann et al. (2001)
In vitro human skin models	302	–	5	Lehmann et al. (2007)
7-Dehydrocholesterol	295	315	1.7	Olds et al. (2010)

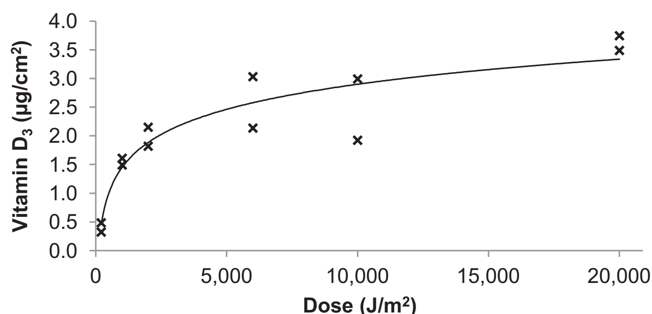


Fig. 3. Production of vitamin D_3 in pig skin at different delivered doses of UVB at 296 nm.

7-DHC (Holick, 1981). In this study the epidermis and dermis were both exposed, as only the subcutaneous fat was removed. From the logarithmic fit it is estimated that the maximum possible production of vitamin D_3 has not been reached, and higher doses would give a higher content of vitamin D_3 in the pig skin. The content of 7-DHC in unexposed skin samples was determined to be 79 ± 6 μg/cm^2 (n = 3). The highest obtained content of vitamin D_3 in the pig skin was between 3.5 and 4 μg/cm^2, which was approx. 4% of the 7-DHC content in unexposed skin. Others have used higher doses of UVB at the same wavelength. MacLaughlin et al. (1982) exposed surgically obtained human skin to different doses of UV in the interval 10,000–300,000 J/m^2 using a wavelength of 295 nm. The results also seem to follow a logarithmic curve where the maximum was not reached even though the highest dose used was 300,000 J/m^2. At this point approx. 70% of the initial 7-DHC had been converted to preD_3 (MacLaughlin et al., 1982). Furthermore, Takada et al. (1979) exposed rat skin with doses of 1530 J/m^2, 3060 J/m^2, 6120 J/m^2, 9180 J/m^2 and 12,240 J/m^2 (using a UV lamp, 280–310 nm). The amount of vitamin D_3 increased linearly with doses. Two studies have reported the results of exposure of *in vitro* human skin models to different doses of UV in the interval 0–4500 J/m^2 using a wavelength of 300 nm. In the first case the vitamin D_3 content increased linearly with doses (Lehmann, Genehr, Knuschke, Pietzsch, & Meurer, 2001), but in the second case the content increased linearly with the dose up to 3000 J/m^2, where it reached a plateau, and stayed constant up to 4500 J/m^2, thereby also following a logarithmic pattern (Lehmann, Knuschke, & Meurer, 2007).

In general there is agreement that a higher dose of UV will result in a higher content of vitamin D_3, and at some point a plateau will be reached thereby following a logarithmic pattern.

3.3. Production of vitamin D_3 is not influenced by total irradiation and exposure time at a constant dose

The effect of five different total irradiation levels at three different wavelengths was investigated at a constant dose, by using the inverse relationship between total irradiation and exposure time. The results for the production of vitamin D_3 are shown in Table 3. For each of the wavelengths, one-way ANOVA was performed, testing the hypothesis of no difference between the different levels of total irradiation used.

Table 3
The content of vitamin D_3 after exposure to a dose of 300 J/m^2 (295–302 J/m^2) at 292, 296 and 300 nm at five different levels of total irradiation (0.1–43 W/m^2).

Wavelength (nm)	Total irradiation (W/m^2)	Vitamin D_3[a] (μg /cm^2)		*P*-value[b]
292	0.1	0.57	0.41	0.62
	3.0	0.46	0.36	
	27.2	0.46	0.45	
	43.0	0.53	0.57	
296	0.1	0.31	0.83	0.96
	3.0	0.43	0.63	
	8.4	0.63	0.61	
	14.8	0.53	0.58	
300	0.1	0.47	0.58	0.06
	2.4	0.71	0.77	
	11.4	0.56	0.43	
	26.8	0.57	0.52	

[a] Each exposure was repeated twice.
[b] *P*-values from one-way ANOVA, testing for no differences between total irradiation.

From the results it can be seen that it is possible to deliver a specific dose of UVB over a long or short time period and achieve the same level of vitamin D_3.

3.4. Application in food production

The maximum achieved content of vitamin D_3 was 3.5–4 μg/cm^2 pig skin, and a content of vitamin D_3 in pig skin of 0.5 μg/cm^2 can be achieved with UV-LEDs by exposure for seven seconds.

Vitamin D_3 in a food product containing pig skin can be tailored by adjusting the applied dose of UVB. For example, a pork loin with a content of 5 μg vitamin D_3/100g would only require a content of 0.15 μg/cm^2 pig skin, assuming a skin surface area of 200 cm^2 and a weight of 600 g. The exposure time can be freely selected to fit into an existing production line, as the vitamin D content at a specific dose is independent of the inversely related parameters; total irradiation and exposure time.

The photo-degradation products of preD_3 are tacysterol$_3$ and lumisterol$_3$ (MacLaughlin et al., 1982). In the blood, vitamin D is transported bound to DBP (vitamin D-binding-protein) (Dueland, Blomhoff, & Pedersen, 1990; Smith & Goodman, 1971). Lumisterol$_3$ has no affinity, and tachysterol$_3$ has a very low affinity for DBP which is why its presence in food will not influence the transport of vitamin D_3 in the circulation (Holick, 1981). According to an EFSA opinion on vitamin D_2 enriched foods, it is not necessary to

include tachysterol$_2$ in the product specifications when the content in the final food product is at or below 0.93 µg/100g (EFSA NDA Panel, 2014). For this reason, analysis of tachysterol$_3$ should be included in future studies aiming to utilise UV-exposure to produce vitamin D – enriched food products.

UV-LEDs as light sources are applicable due to their compact design and low energy consumption. Furthermore, LEDs can be implemented in industrial settings, while the traditional bulky sources of narrowband UV are only practical for laboratory use. LEDs allow spectral control of the emitted light, and can be easily integrated into electronic systems for automation. Safety rules and energy consumption are the first challenges that need to be addressed when installing UV-light sources in a food production facility. LED technology can provide dust- and moisture – proof solutions, as well as ensuring great mechanical stability, and a lack of toxic compounds. LEDs also produce minimal radiant heat, compared to other UVB-light sources, so unwanted surface heating is avoided (Souza, Yuk, Khoo, & Zhou, 2015).

Moreover, LED systems have longer expected lifetimes, lower energy consumption, and lower maintenance costs than other UVB-light sources. However, up-front costs of installing an UVB-LED based lighting system are currently high. However, costs are expected to fall in the near future (Bergh, 2004), and LED performance is expected to continue to improve (Nishida, Saito, & Kobayashi, 2001; Yam & Hassan, 2005).

Future projects should assess the relevant dose needed to produce, e.g., pork loin, roast pork with crackling, fried pork, and pork crackling with an enhanced content of vitamin D$_3$.

4. Conclusion

The optimal wavelength for the production of vitamin D$_3$ in pig skin irradiated with LED-UV was determined to be 296 nm.

At 296 nm the effect of dose on the production of vitamin D$_3$ in pig skin follows a logarithmic curve. The maximum applied dose of 20 kJ/m^2 resulted in a vitamin D$_3$ content of 3.5–4 µg/cm^2.

An increase in content of vitamin D$_3$ in pig skin can be obtained by a specific dose, which may either be given at low irradiation and long exposure time, or high irradiation combined with a short exposure time.

Food products containing pork skin may be enriched by LED-UVB exposure to increase the content of vitamin D$_3$.

Acknowledgments

The authors thank Simone Santos Faria for her huge effort in the laboratory. The Technical University of Denmark, Denmark, and a grant from the European Commision under its Seventh Framework Programme (ODIN; grant agreement no. 613977) funded the project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.05.155.

References

Bergh, A. (2004). Blue laser diode (LD) and light emitting diode (LED) applications. Physica Status Solidi (A) Applied Research, *201* (12), 2740–2754.

Black, L. J., Seamans, K. M., Cashman, K. D., & Kiely, M. (2012). An updated systematic review and meta-analysis of the efficacy of vitamin D food fortification. *The Journal of Nutrition, 142*(6), 1102–1108.

Bunker, J., & Harris, R. S. (1937). Precise evaluation of ultraviolet therapy in experimental rickets. *New England Journal of Medicine, 216*(4), 165–169.

Burild, A., Frandsen, H. L., Poulsen, M., & Jakobsen, J. (2014). Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. *Journal of Separation Science, 37*(19), 2659–2663.

Burild, A., Frandsen, H. L., Poulsen, M., & Jakobsen, J. (2015). Tissue content of vitamin D3 and 25-hydroxy vitamin D3 in minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D3. *Steroids, 98*, 72–79.

Burild, A., Lauridsen, C., Faqir, N., Sommmer, H. M., & Jakobsen, J. (2016). Vitamin D3 and 25-hydroxy vitamin D3 in pork and their relationship to the vitamin D status in pigs. *Journal of Nutritional Science, 5*, e3.

Dueland, S., Blomhoff, R., & Pedersen, J. I. (1990). Uptake and degradation of vitamin D binding protein and vitamin D binding protein-actin complex in vivo in the rat. *Biochem. J., 267*(3), 721–725.

EEC (2004). List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. *Official Journal of the European Union, C50*(1), 1–144.

EFSA NDA Panel (2014). EFSA panel on dietetic products, nutrition and allergies. Scientific Opinion on the safety of vitamin D-enriched UV-treated baker's yeast. *EFSA Journal, 12*(1), 3520–3539.

Food and Drug Administration (2012). Food additives permitted for direct addition to food for human consumption; Sec. 172.381 Vitamin D2 bakers yeast. *Federal Register, 77*(168), 52228–52232.

Holick, M. F. (1981). The cutaneous photosynthesis of previtamin D3: A unique photoendocrine system. *Journal of Investigative Dermatology, 77*(1), 51–58.

Holick, M. F., MacLaughlin, J. A., Clark, M. B., Holic, S. A., Potts, T. J., Anderson, R. R., ... Elias, P. (1980). Photosynthesis of previtamin D3 in human skin and the physiologic consequences. *Science, 210*(4466), 203–205.

Institute of Medicine (2011). *Dietary reference intakes for vitamin D and calcium*. Washington DC: The National Academies Press.

ISO (2005). *ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories*.Geneva, Switzerland: ISO Central Secretariat.

Jakobsen, J., Jensen, S. K., Hymøller, L., Andersen, E. W., Kaas, P. 312, Burild, A., & Jäpelt, R. B. (2015). Artificial ultraviolet B light exposure increases vitamin D levels in cow plasma and milk. *Journal of Dairy Science, 98*(9), 6492–6498.

Jasinghe, V. J., & Perera, C. O. (2006). Ultraviolet irradiation: The generator of vitamin D2 in edible mushrooms. *Food Chemistry, 95*(4), 638–643.

Knudson, A., & Benford, F. (1938). Quantitative studies of the effectiveness of ultraviolet radiation of various wave-lengths in rickets. *Journal of Biological Chemistry, 124*, 287–299.

Kobayashi, T., Hirooka, M., & Yasumura, M. (1976). Effect of wavelength on the ultraviolet irradiation of 7-dehydrocholesterol. *Vitamins, 50*, 185–189.

Kobayashi, T., & Yasumura, M. (1973). Studies on the ultraviolet irradiation of provitamin D and its related compounds III. Effect of wavelength on the formation of potential vitamin D2 in the irradiation of ergosterol by monochromatic ultraviolet rays. *Journal of Nutritional Science and Vitaminology, 19*(2), 123–128.

Kristensen, H. L., Rosenqvist, E., & Jakobsen, J. (2012). Increase of vitamin D2 by UV-B exposure during the growth phase of white button mushroom (*Agaricus bisporus*). *Food & Nutrition Research, 56*(na), 1–7.

Lehmann, B., Genehr, T., Knuschke, P., Pietzsch, J., & Meurer, M. (2001). UVB-induced conversion of 7-dehydrocholesterol to 1α,25-dihydroxyvitamin D3 in an in vitro human skin equivalent model. *J. Invest. Dermatol., 117*(5), 1179–1185.

Lehmann, B., Knuschke, P., & Meurer, M. (2007). The UVB-induced synthesis of vitamin D3 and 1α,25-dihydroxyvitamin D3 (calcitriol) in organotypic cultures of keratinocytes: Effectiveness of the narrowband Philips TL-01 lamp (311 nm). *Journal of Steroid Biochemistry and Molecular Biology, 103*(3–5), 682–685.

MacLaughlin, J. A., Anderson, R. R., & Holick, M. F. (1982). Spectral character of sunlight modulates photosynthesis of previtamin D3 and its photoisomers in human skin. *Science, 216*(4549), 1001–1003.

Maughan, G. (1928). Ultra-violet wavelengths valuable in the cure of rickets in chickens. *American Journal of Physiology, 87*(2), 381–398.

Nishida, T., Saito, H., & Kobayashi, N. (2001). Efficient and high-power AlGaN-based ultraviolet light-emitting diode grown on bulk GaN. *Applied Physics Letters, 79* (6), 711–712.

Nordic Nutrient Recommendation (2014). *Nordic Nutrition Recommendations 2012*. Nordic Council of Ministers. 5(11), 1. http://dx.doi.org/10.6027/Nord2014-002. Assessed 15.10.15.

Norman, A. (1979). *Vitamin D: The calcium homeostatic steroid hormone*.New York: Academic Press.

O'Mahony, L., Stepien, M., Gibney, M. J., Nugent, A. P., & Brennan, L. (2011). The potential role of vitamin D enhanced foods in improving vitamin D status. *Nutrients, 3*(12), 1023–1041.

Okano, T., Yasumura, M., Mizuno, K., & Kobayashi, T. (1978). In vivo and in vitro conversion of 7-dehydrocholesterol into vitamin D3 in rat skin by ultraviolet ray's irradiation. *Journal of Nutritional Science and Vitaminology, 24*(1), 47–56.

Olds, W. J., Lucas, R. M., & Kimlin, M. G. (2010). Action spectrum for vitamin D synthesis. In *UV radiation and its effects – 2010, NIWA UV workshop. 7–9 April 2010. NIWA Information Series No. 77. Copthorne Hotel, Queenstown, New Zealand* (pp. 106–107).

Pedersen, A., Christensen, T., Matthiesen, J., Knudsen, V. B., Rosenlund-Sørensen, M., Biltoft-Jensen, A., ... Fagt, S. (2015). *Dietary habits in Denmark 2011–2013. Main results*. . ISBN 978-87-93109-39. Søborg.

Saxholt, E., Christensen, A. T., Møller, A., Hartkopp, H. B., Ygil, K. H., & Hels, O. H. (2008). *Danish food composition databank, revision 7*. Department of Nutrition, National Food Institute, Technical University of Denmark. http://www.foodcomp.dk/v7/fvdb_search.asp. Accessed 15.10.15.

Smith, J. E., & Goodman, D. S. (1971). The turnover and transport of vitamin D and of a polar metabolite with the properties of 25-hydroxycholecalciferol in human plasma. *The Journal of Clinical Investigation, 50*(10), 2159–2167.

Souza, C. D., Yuk, H., Khoo, G. H., & Zhou, W. (2015). Application of light-emitting diodes in food production, postharvest preservation, and microbiological food safety. *Comprehensive Reviews in Food Science and Food Safety, 14*, 719–740.

Sternberg, J. C., Stillo, H. S., & Schwendeman, R. H. (1960). Spectrophotometric analysis of multicomponent systems using least squares method in matrix form. Ergosterol irradiation system. *Analytical Chemistry, 32*(1), 84–90.

Takada, K., Okano, T., Tamura, Y., Matsui, S., & Kobayashi, T. (1979). A rapid and precise method for the determination of vitamin D3 in rat skin by high-performance liquid chromatography. *Journal of Nutritional Science and Vitaminology, 25*(5), 385–398.

Yam, F. K., & Hassan, Z. (2005). Innovative advances in LED technology. *Microelectronics Journal, 36*(2), 129–137.

Supplementary Online Material for

Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin.

Line Lundbæk Barnkob[a], Aikaterini Argyraki[b], Paul Michael Petersen[b] and Jette Jakobsen[a]*

[a] National Food Institute, Technical University of Denmark, Denmark
[b] Department of Photonics Engineering, Technical University of Denmark, Denmark

* Corresponding author
E-mail address: jeja@food.dtu.dk (Jette Jakobsen)

This PDF file includes for

Materials and methods

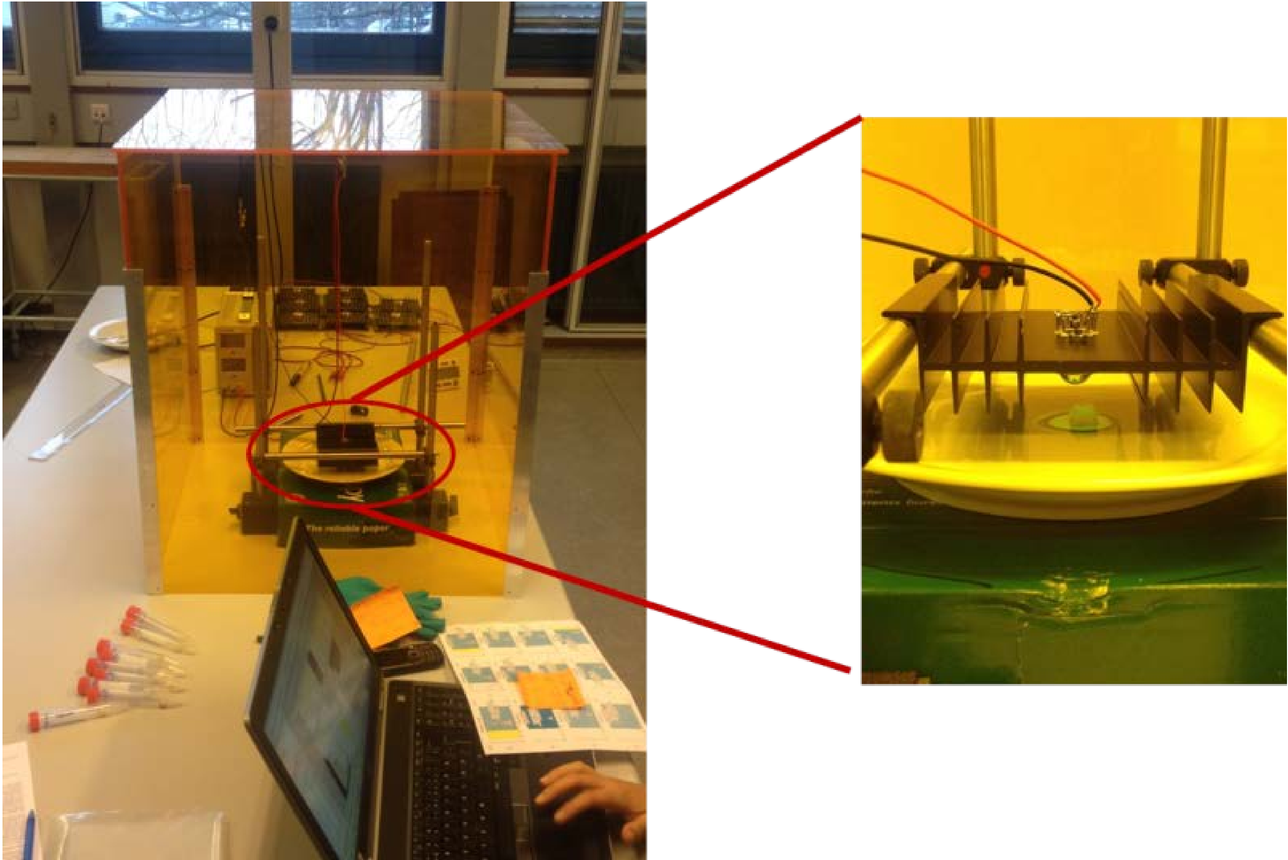
2.3. Experimental design

S1. Setup of the UV-LED equipment
Figure S1

S2. The exact UV-LED settings used in the experiments
Table S1-S3

19 **1. Setup of the UV-LED equipment**

20



21

22 **Figure S1.** The setup for exposure of samples with UV light, using LEDs. The orange box
23 is opaque to UV light and is used for protection from the irradiation. The distance between
24 the light source and the surface of the sample was kept constant in all experiments.

25

26

2. The exact UV-LED settings used in the experiments

27 **Table S1.** For the determination of the effect of wavelength on the production of vitamin D_3
28 in pig skin the UV-LEDs were operated in constant current mode. The effect was
29 determined at two different doses 300 J/m^2 and 7000 J/m^2. The current, total irradiance,
30 exact dose and exposure time for each of the wavelengths are given for each of the doses.

Dose	300±3 J/m2			7000±3 J/m2		
Central wavelength (nm)	Current (mA)	Total irradiance (W/m2)	Exposure (sec)	Current (mA)	Total irradiance (W/m2)	Exposure (sec)
281	100	9.8	31	200	21.2	330
285	100	10.6	28	200	23	304
292	200	11.6	26	200	14	500
296	500	11.4	26	200	3.2	2188
300	300	11.4	26	200	8.2	854
306	400	10.2	29	200	6	1167
310	400	9.4	32	200	5.6	1250
313	400	9.4	32	200	5.6	1250
318	300	12.4	24	200	9	778
330	600	8.2	37	200	2	3500
336	200	11.4	26	200	11.2	625
338	200	9.8	31	200	9.6	729

31

32 **Table S2.** The settings of the UV-LEDs for the determination of the effect of dose.

Central wavelength (nm)	Current (mA)	Total irradiance (W/m2)	Dose (J/m2)	Exposure (sec)
296	600	14.8	207	14
296	600	14.8	1008	68
296	600	14.8	2001	135
296	600	14.8	6002	405
296	600	14.8	10004	675
296	600	14.8	20007	1350

33

34

35 **Table S3**. The specific LED settings used for each exposure, to determine the effect of
36 total irradiance/exposure time on the production of vitamin D_3 in pig skin.

Wavelength (nm)	Current (mA)	Total irradiance (W/m^2)	Dose (J/m^2)	Exposure (sec)
292±4	8	0.1	300	3000
	70	3.0	300	100
	400	27.2	299	11
	600	43.0	301	7
296±4	30	0.1	300	3000
	200	3.0	300	100
	400	8.4	302	36
	600	14.8	296	20
300±4	10	0.1	300	3000
	100	2.4	300	125
	300	11.4	296	26
	600	26.8	295	11

37

Paper II

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

Line Lundbæk Barnkob[1], Paul Michael Petersen[2], Jens Peter Nielsen[3] and Jette Jakobsen[1]

[1] Technical University of Denmark, National Food Institute, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark

[2] Technical University of Denmark, Department of Photonics Engineering, Frederiksborgvej 399, 4000 Roskilde, Denmark

[3] University of Copenhagen, Division for Production, Nutrition and Health, Grønnegårdsvej 2, 1870 Frederiksberg C, Denmark

Jette Jakobsen

jeja@food.dtu.dk

Funding

A grant from the European Commission under its Seventh Framework Programme (ODIN; grant agreement no. 613977) and the Technical University of Denmark funded the project.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Vitamin D deficiency is a recognized problem in Europe; this can be minimized by fortifying a broader range of foods. Our aim was to investigate the potential for enhancing the vitamin D content of pork from pigs raised in indoor facilities, by exposing the pigs to UVB for a period just before slaughter. Three groups of six pigs were exposed to 0, 0.7 or 1 SED/day for 28 days. A fourth group was exposed to 2 SED; this treatment was not completed due to mild erythema. The highest increase of vitamin D_3 was achieved with 1 SED; the vitamin D_3 content in loin was 3.7 ng/g; more than a factor of 2 higher compared to previously reported results from studies using 2000 IU/kg feed, the maximum allowed level in Europe. This is the first time an increase in the vitamin D content of pork has been reported as a result of using artificial UVB-exposure of slaughter pigs in indoor facilities. However, the maximum production of vitamin D was probably not reached as a linear relationship between UVB dose and vitamin D content was found; therefore, the UVB-lighting method described still calls for further investigation to realise its full potential to enhance vitamin D in pork.

Keywords

Vitamin D; UVB; Pork; Pigs

Introduction

On annual basis around 13 % of the European population is experiencing vitamin D deficiency (vitamin D status < 30 nmol/L) and 40 % vitamin D insufficiency (vitamin D status < 50 nmol/L) [1]. Although the recommended intake of vitamin D is between 10-20 µg/day [2, 3], the estimated intake is only around 3-7 µg/day[4]. It has been shown that vitamin D intake may be increased by effective food-based strategies [5, 6]. A feasible strategy is to fortify a broader range of foods and/or increase the natural content of vitamin D in foods, e.g. fatty fish, eggs and meat [4, 7, 8].

Adding vitamin D to the final food product e.g. milk dairy products, and breakfast cereals is the traditional way of fortifying [9]. An alternative is using the natural mechanisms to enhance the vitamin D content in foodstuff;. Like humans, livestock are able to produce vitamin D_3 in the skin when exposed to UVB light (290-320 nm) either artificially or from the sun [10–15]. Also when adding more vitamin D to the livestock feed the vitamin D content in the final food product will increase [16, 17].

Vitamin D exists in two forms, vitamin D_2 and vitamin D_3. Vitamin D and 25-hydroxyvitamin D (25(OH)D), the major metabolite of vitamin D, is naturally found in products of animal origin; although there are other metabolites only 25(OH)D is so far included when determining the total vitamin D content in food [18, 19].

EU has maximum limits for adding vitamin D to feed. For pigs this limit is 2000 IU/kg feed (Commission Directive 91/248/EEC), that is reported to produce lean pork loin with a content of 1.2 ng vitamin D_3/g and liver with a content of 8.4 ng vitamin D_3/g [17]. In Denmark, the pigs are not fed with the maximum allowed level of vitamin D as the recommended level for pigs (30-100 kg) is 400 IU/kg feed [20], that result in lean pork with a vitamin D_3 content of 0.24 ng/g [21]. This is in contrast to earlier findings from our research group where a vitamin D_3 content of 5-11 ng/g and 42 ng/g was measured in muscle tissue and in liver, respectively, from mini-pigs after they had been exposed to UVB light; the daily exposure was similar to 10-20 min summer sun at 55 °N, for 120 days [10].

Already in 1924 it was shown that pigs produce vitamin D when exposed to sunlight [22]; and in 1925 it was reported that indoor livestock production reduces the vitamin D content in animal products for human consumption [23]. A recent study shows that the vitamin D content will increase in the meat when pigs are exposed to sunlight during the summer; specifically1 hour of summer sunlight per day for14 days before slaughter resulted in lean pork loin with a content of 7.2 ng vitamin D_3/g [13]. However during winter if above or below 35 °N and 35 °S, respectively, no cutaneous vitamin D will be produced [24]. Giving pigs' access to an outdoor area will therefore not alleviate the problem with human vitamin D deficiency, which is a problem, especially in winter.

The aim of this study was to investigate the potential to enhance the vitamin D content of pork from pigs raised in indoor facilities, by exposing the pigs to UVB-light for a period just before slaughter.

Materials and methods

Animals and test environment

The study included 24 slaughter pigs, all females and fair-skinned, of the Danish breed LYD (Landrace, Yorkshire and Duroc); purchased from Askelygård, Roskilde, Denmark. At delivery the pigs had a mean weight of 50 kg (range 44-79 kg). All pigs were clinically healthy at arrival.

The pigs were allocated at random into 4 groups of 6 and placed in pens of 3x4.5 m, which were separated by UV-impenetrable walls.

The pigs were fed standard feed with a vitamin D_3 content of 420 IU/kg (Danish Top S1 611+3+5, Danish Agro, Karise, DK). The feed was administered twice a day. In the last 28 days of the trial the amount was up to 1 kg per pen per feeding. Water was offered ad libitum, and fresh straw was provided every day. The stable was lit by fluorescent tubes from 8:00 to 14:30 during the entire study. The fluorescent tubes did not emit any UVB light.

The trial ran from November 2016 to February 2017. The pigs were kept at the research facility for large animals at University of Copenhagen (Rørrendegård, Taastrup, Denmark).

Study design and sample size determination

The UVB-treatment was a parallel study with three treatment groups and one control group; it lasted 28 days. Based on sample size calculation with α=5 %, 1-β=0.8, SD=24 % [16] and an expected increase of 50 %, a group size of four was needed; however six were chosen to be on the safe side.

UVB-exposure

In each of the pens housing a treatment group, four UVB tubes (Lucky Reptile UV sun T5, 54 W tubes, luckyreptile.com, Germany) were placed at a height of approximately 180 cm above the floor. Normal stable armatures were used, but without plastic covers, as they absorbed the UVB-light. The pigs in the 3 treatment groups were gradually adapted to UVB light over the first 8 days of exposure. The doses are stated in units of standard erythemal dose (SED) where 1 SED is equivalent to 100 J/m^2 erythemal dose [25]. During the first 7 days the 3 treatment groups were given the same doses: day 1-2, 0.3 SED; day 3-5, 0.6 SED; day 6-7, 0.7 SED. From day 9 the remaining 2 groups were exposed to the planned doses of 1.0 SED and 2.0 SED, respectively, while the third group continued with 0.7 SED. The control group received no UVB exposure. The pigs were slaughtered on day 29, after 28 days of UVB-exposure.

UVB measurements

At DTU Fotonik, Department of Photonics Engineering, Technical University of Denmark, a full spectrum of the irradiance was recorded for one tube, scanning from 200 nm to 700 nm, with 1 nm increment (EOP146 detector probe, Instrument Systems-CAS140CT). The tube emitted UVB from 286 nm to 320 nm, and had a maximum at 312 nm. The full spectrum is shown in Online Resource 1.

The erythemal effective irradiance was calculated using the measured spectrum and the erythema reference action spectrum [25] which couples irradiance to the development of erythema. A figure of the measured spectrum with the erythema reference action spectrum superimposed is shown in Online Resource 2. For measurements in the stable a handheld ILT 1400-BL photometer equipped with a SEL005/TLS312/TD detector (International Light Technologies, Peabody, MA) was used to measure the irradiance at 16 positions at two distances from the floor; 30 cm and 60 cm, to illustrate pigs lying and standing. The average of the 32 measurements combined with the calculated erythemal effective irradiance was used to assess the exposure times of 150, 214, and 427 minutes, in order to achieve the target doses of 0.7, 1 and 2 SED, respectively.

Weighing

The pigs were weighted 7 days before the UVB treatment started and just before slaughter.

Slaughtering process

The 24 pigs were electrically stunned, then stunned with a captive-bolt pistol, pithed and then hoisted by one leg and stuck. After bleeding, the pigs were scalded and dehaired in a scalding tub, followed by singeing using a hand-held gas torch. After singeing, black deposits and singed hairs were scraped off with a knife and washed off before evisceration; the liver was collected and kept at -20 °C until further treatment. The carcasses were cut in half and hung in cold storage overnight.

Sampling and homogenization

The day after slaughter, half of each carcass was divided into primal cuts and kept at -20 °C until further treatment within four days. The middle third of the pork loin with rind (Longissimus dorsi with subcutaneous fat and skin; approximately 1.2 kg) was carefully divided into lean meat, subcutaneous fat and rind. Before analysis the liver, lean meat and subcutaneous fat was homogenized (Tecator 1094 Homogenizer (Foss Tecator, Höganäs, Sweden) and Bamix® SliceSy®, respectively). The rind was divided into 20 strata, and from each stratum a random small biopsy (diameter: 4 mm) was taken and combined into one sample.

Vitamin D analysis

The analytical method used on tissue samples, which included vitamin D_3, $25(OH)D_3$, vitamin D_2 and $25(OH)D_2$, was developed by combining and optimizing different parts of two published methods for the quantification of vitamin D_3 and $25(OH)D_3$ in serum and tissue [26, 27]. A full description of the method is available in Online Resource 3. In short, the food samples were saponified over night at room temperature, clean-up by liquid-liquid extraction and solid phase

extraction. For quantification on LC-MS/MS vitamin D metabolites were derivatized by 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). In serum samples the protein was precipitated and clean-up by solid phase extraction.

The precision of the method was < 10 % in food and < 5 % in serum. Furthermore the correctness of the method was checked by analyses of certified reference materials (Milk powder, CRM421, IRMM, Geel, Belgium) and recovery of added vitamin D. The result was 151 ng vitamin D_3/g ± 2.4 % (n=8) which was within the certified values of 0.143 ± 0.008 mg/kg, while the recovery for vitamin D_3, vitamin D_2, 25(OH)D_3 and 25(OH)D_2 was 90-127 % (n=9). All of the analyses were conducted in laboratory environment accredited to perform the analyses according to ISO17025 [28].

Content of fat

The fat content was determined gravimetrically using a Schmid-Bondynski-Ratslaff (SBR) method [29]. The sample was boiled in hydrochloric acid, ethanol was then added and the lipids were extracted using diethyl ether:petroleum ether (1:1) that was subsequently evaporated and the fat was weighed.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey tests was used to determine significant difference in the vitamin D content between the individual groups using 95 % family-wise confidence interval. The means were log transformed to fulfill the assumption of equal variance and normal distribution; except for vitamin D_2 in liver. The 2 SED group was excluded from the analyses as the treatment was not completed. One-way ANOVA was used to test for difference in fat content between the groups. The statistical analyses were performed in R [30] using RStudio [31]. Association between vitamin D status and content of vitamin D in other parts of the pigs were assessed with Pearson's correlation coefficient using the Data Analysis function in Excel (version 2010, Microsoft, Redmond, WA). Data is reported as mean ± standard deviation (SD).

Results

Clinical findings

No effect on health or behavior was observed in three of the groups: control, 0.7 SED and 1 SED. The pigs in the 2 SED group developed mild erythema on the ears and backs after 10 days with the dose of 2 SED, i.e. day 19, therefore, the UVB treatment was immediately ceased for this group. The veterinarian did not find the redness severe and the general well-being and behavior of the pigs were normal; the pigs were therefore kept alive until the end of the trial and slaughtered together with the other pigs at day 29. Thus this group did not complete the treatment and was left out from further analyses.

Content of vitamin D and fat

The measured content of vitamin D_3, 25(OH)$D_{3,}$ vitamin D_2, 25(OH)D_2 is displayed in Table 1 from the three groups that completed the treatment. The main finding was that the treatment with 1 SED resulted in the highest content of vitamin D_3; specifically the content in rind, subcutaneous fat, lean meat, and liver was 316 ng/g, 120 ng/g, 3.7 ng/g, and 29 ng/g, respectively.

Table 1. Content of the four vitamers in rind, subcutaneous fat and lean meat from pork loin, in liver and in serum, and fat percentage for the samples from loin from pigs treated with different doses of UVB for 28 days.

	0 SED		0.7 SED		1 SED	
	Mean	SD	Mean	SD	Mean	SD
Rind						
Fat (%)	11.7[a]	2.3	11.0[a]	2.4	12.2[a]	2.8
Vitamin D_3 (ng/g)	1.7[a]	0.2	146[b]	76	316[c]	159
25(OH)D_3 (ng/g)	2.0[a]	1.0	12[b]	4	21[c]	8
Vitamin D_2 (ng/g)	0.5[a]	0.1	0.8[b]	0.2	1.1[b]	0.3
25(OH)D_2 (ng/g)	0.9	0.3	< 0.5		< 0.5	
Subcutaneous fat						
Fat (%)	77.9[a]	5.5	74.5[a]	5.1	78.4[a]	4.7
Vitamin D_3 (ng/g)	4.1[a]	1.1	66[b]	21	120[c]	56
25(OH)D_3 (ng/g)	0.9[a]	0.2	4.1[b]	0.8	6.9[c]	1.8
Vitamin D_2 (ng/g)	1.7[a]	0.9	2.6[b]	1.0	2.8[b]	0.7
25(OH)D_2 (ng/g)	< 0.5		< 0.5		< 0.5	
Lean meat						
Fat (%)	1.9[a]	0.6	1.8[a]	0.3	1.5[a]	0.2
Vitamin D_3 (ng/g)	0.2[a]	0.03	2.3[b]	0.4	3.7[c]	1.0
25(OH)D_3 (ng/g)	0.3[a]	0.08	1.6[b]	0.2	2.4[c]	0.8
Vitamin D_2 (ng/g)	0.3[a]	0.08	0.4[b]	0.1	0.5[b]	0.1
25(OH)D_2 (ng/g)	< 0.5		< 0.5		< 0.5	
Liver						
Vitamin D_3 (ng/g)	0.8[a]	0.3	14.9[b]	3.8	29[c]	9
25(OH)D_3 (ng/g)	0.8[a]	0.2	4.9[b]	0.8	8.3[c]	2.4
Vitamin D_2 (ng/g)	0.7[a]	0.6	2.3[b]	0.3	2.7[b]	0.4
25(OH)D_2 (ng/g)	< 1.0		< 1.0		< 1.0	
Serum						
Vitamin D_3 (ng/ml)	0.8[a]	0.3	17[b]	4	31[c]	9
25(OH)D_3 (ng/ml)	11[a]	3	52[b]	8	95[c]	25
Vitamin D_2 (ng/ml)	0.4[a]	0.2	0.8[b]	0.1	1.0[b]	0.3
25(OH)D_2 (ng/ml)	5.1[a]	1.3	2.3[b]	0.2	2.2[b]	0.4

[a,b,c] Mean values within a row with unlike superscript were significantly different (One-way ANOVA with Tukey test, $p < 0.05$).

There was a significant association between the serum 25(OH)D_3 (vitamin D status) and the vitamin D_3 content in the other tissues of the pig (Pearson's R 0.92-0.98). A table with the exact values is available in Online Resource 4.

There was also a significant association between the UVB dose and the content of vitamin D_3 and 25(OH)D_3 in all parts of the pig (Pearson's R 0.96-1.00). The vitamin content as a function of UVB dose is shown in Online Resource 5.

Weight

The average live weight at slaughter was 112±6 kg; equal to the average live weight of slaughter pigs in Denmark. The average weight gain during the UVB treatment was 22±3 kg; there were no significant difference between groups.

Discussion

The main goal of this study was to investigate the potential to enhance the vitamin D content of pork from pigs raised in indoor facilities, by exposing pigs to artificial UVB for a period just before slaughter.

This is the first study that reports data on the effect of different UVB-exposure levels in slaughter pigs. The doses of 0.7 SED, 1 SED and 2 SED were chosen to simulate approximately 7 to 20 minutes of midday sun exposure during summertime in Denmark (56 °N) [32]. We had planned to investigate the effect of increasing the dose to a level of 2 SED, but unfortunately the treatment with 2 SED had to be stopped, due to mild erythema (see Clinical findings in the Results section). However, the results for 0, 0.7 and 1 SED showed that the vitamin D_3 and 25-hydroxyvitamin D_3 content in the analysed parts of the pig was linearly associated with the daily exposure time with a Pearson coefficient > 0.96. This indicates that the maximum production of vitamin D has not been reached using 1 SED as the vitamin D production after exposure to UVB has been shown to be non-linear in humans and hens [33, 34].

In an earlier study from our research group, mini-pigs did not develop erythema when exposed to a UVB dose of 0.9 SED for 84 days followed by 1.8 SED for 35 days [10]. The adaption period in the present study was 5, 7 and 9 days for the groups 0.7 SED, 1 SED and 2 SED, respectively. We therefore assume that the mild erythema in the 2 SED group was due to the shorter adaption, which was used in the present study.

The high content of vitamin D_3 in skin is expected to decline if the period between the last UVB exposure and slaughter is extended beyond the 1 day in the present study; in rats it has been shown that vitamin D_3 is released from the skin into the circulation for up to three days after UVB exposure and around 70 % of the possible vitamin D_3 is released within the first day [35].

Kolp et al. [14] investigated a single level of UVB-exposure on pigs (1.25 SED/week[1]) for 14 weeks before slaughter with the aim of investigating the effect on calcium and bone metabolism for the purpose of studying the health effects

[1] 0.96 MED (minimal erythemal dose) where 1 MED is 125 J/m^2 according to their reference and thereby equal to 1.25 SED.

for the pigs. However, besides 25(OH)D_3 in serum, they also included analyses of vitamin D_3 in skin. They reported no effect of the UVB-exposure on the content of vitamin D_3 in skin, but they did show an increase in serum 25(OH)D_3. Compared to our study, they used less than 0.2 SED daily, which could be the reason for the different results.
Another strategy which has been investigated is the exposure of pigs to sunlight during summer in order to increase the vitamin D content in pork [13]; this study includes a control and one treatment group of 1 hour of summer sunlight per day for14 days before slaughter. An effect of sunlight was shown for content of vitamin D_3 and 25(OH)D_3 in lean pork loin, and for 25(OH)D_3 in subcutaneous fat, but no increase in the vitamin D_3 content in the subcutaneous fat was observed. Larson-Meyer et al. [13] suggest that they reached the limit of how much vitamin D_3 can be stored in the adipose tissue, however, in light of our findings this seems not to be the explanation.

During winter if above or below 35 °N and 35 °S, respectively, no cutaneous vitamin D will be produced [24]; giving pigs' access to an outdoor area will therefore not alleviate the problem with human vitamin D deficiency, which is a problem, especially in winter.

Effect of UVB exposure compared to effect of feeding trials

Maximum increase in vitamin D_3 was obtained with a daily exposure of 1 SED; compared to the control group the content in lean meat was increased 19 times. The dose of 1 SED was used in the last 19 of the 28 days the UVB trial lasted and resulted in a content of 3.7 ng/g vitamin D_3 (range: 2.3-5.3 ng/g) in lean meat; this is more than two-fold higher compared to the vitamin D_3 content of 1.2 ng/g loin from pigs fed 2000 IU/kg, which is the maximum allowed vitamin D_3 in feed in Europe [17]. The vitamin D_3 content in subcutaneous fat was found to be 120 ng/g (range 40-240 ng/g) that is 5-20 times higher than formerly reported values of 7.5-7.6 ng/g in subcutaneous fat from pigs fed 2000 IU/kg [16, 17]. In rind, 1 SED resulted in a vitamin D_3 content of 259 ng/g (range 170-530 ng/g) that is 55-170 times higher than the 3 ng/g reported in a feeding study at maximum feeding level [16]. The liver from pigs exposed to 1 SED had a content of 29 ng/g (range 20-45 ng/g) that is 2-5 times higher than the 8.4 ng/g in liver from pigs fed 2000 IU/kg [17]. This content was lower than in liver from mini-pigs exposed to UVB [10], but the duration of the UVB treatment differed, being 120 days for mini-pigs and 28 days in this study.

Similar to vitamin D_3 the content of 25(OH)D_3 was highest in the 1 SED group, and higher in the UVB-exposed pigs compared to the results from feeding trials using 2000 IU/kg feed [16, 17].

Unexpected increase in vitamin D_2

Unexpectedly there was found an increase in the vitamin D_2 content in the UVB treatment groups compared to the control. We suggest that it originates from either feed or straw contaminated with fungi. In another study it is shown that when grass contaminated with fungi is exposed to UVB the ergosterol in the fungi is converted to vitamin D_2 [36]. This would explain why only the groups with UVB-exposure show an increase in vitamin D_2 as the straw and the feed were exposed to the UVB when administered to the pigs and thereby functioned as a source of vitamin D_2 in the UVB exposed groups. We cannot confirm if this is the explanation, as neither the straw nor the feed were analysed for vitamin D metabolites.

Interaction between vitamin D_2 and vitamin D_3

In contrast to vitamin D_2, the control group had the highest content of 25(OH)D_2. The 25(OH)D_2 content in serum from the UVB treated groups were significantly lower than in serum from the control group. Furthermore 25(OH)D_2 was present in rind from the control group but not found in the in rind from the UVB treated groups. To our knowledge this is the first time an increase in vitamin D_3 has been reported to result in a decrease in 25(OH)D_2. Previously, only a decreasing effect of vitamin D_2 intake on 25(OH)D_3 serum concentration has been reported as summarised by Wilson et al. [37]. Lehmann et al. [38] administered vitamin D_2, D_3 or placebo to healthy humans for a period of 8 weeks. They suggested that vitamin D_2 impairs the hydroxylation of vitamin D_3 as it explained that they observed a higher decrease in serum 25(OH)D_3 in the group administered with vitamin D_2 than in the placebo group [38]. However, based on our results we suggest that the hydroxylation of vitamin D_2 and vitamin D_3 is a classic case of competitive inhibition where the substrate present in the highest concentration inhibits the hydroxylation of the substrate with the lower concentration [39].

Effect of UVB exposure on variation in vitamin D content

The within group variation, in vitamin D content, is generally low in feeding trials [16, 17]. The variation in the control group in this study was also low, while the variation in the UVB exposure groups was higher. The relative precision (RSD) for the vitamin D_3 content measured in rind, subcutaneous fat and lean meat from the 1 SED group was 4.3, 1.7

and 1.8 times higher, respectively, compared to RSD from the control group. Similarly, sun exposure causes higher variation [13].

The effect of UVB-exposure on vitamin D content in pork possibly depends on three factors: the height of the pigs; the individual behavior, especially standing or lying; and the effectiveness of the endogenous production of vitamin D_3 in the skin. There was noticeable height difference between the pigs; the height of the individual pigs was however not measured but was estimated to be around 60 cm at arrival and 70 cm at slaughter. The higher the pig, the closer the pig was to the UVB tube and thereby the dose received would be higher giving raise to higher vitamin D production; the same reasoning applies for some pigs standing more than others. Some of the pigs had pigmentation on part of their bodies, this could in theory decrease the effectiveness of the endogenous production of vitamin D [40], though for cows no effect is observed [11].

25(OH)D_3 as biomarker for vitamin D content

One study has shown that there is a linear relationship between the serum 25(OH)D_3 and the vitamin D_3 and 25(OH)D_3 content in meat when vitamin D_3 is added to the feed [17]. In the present study serum 25(OH)D_3 was associated to the content of vitamin D_3 and 25(OH)D_3 in rind, fat, lean meat, liver and serum. This indicates that a model may be set up between serum 25(OH)D_3 and the content of vitamin D_3 and 25(OH)D_3 in pork products. Such a model could be useful in quality control of vitamin D content in pork products, as the analysis of serum 25(OH)D_3 is easier, faster and cheaper compared to analysis of other tissues. Larson-Meyer et al. [13] also found a positive association between serum 25(OH)D_3 concentrations and vitamin D_3 in loin (R 0.63) although not as high as ours (R 0.96). They also found a positive association with 25(OH)D_3 in loin (R 0.83). The reason for the stronger association shown in our study might be due to the difference in analytical assays for serum 25(OH)D_3, namely RIA in Larson-Meyer et al. [13] and a chemical assay in this study. A concentration dependent difference has been shown between the two analytical principles of quantification ; the linear correlation between the two is segmented with a shift around 50 nmol/L[41, 42].

Effect of UVB exposure on weight gain

UVB exposure did not have an effect on the weight gain of the pigs. The same was found in studies where vitamin D status was followed in pigs exposed to 1 hour of sunlight for a total of 28 days, and in pigs exposed for 14 weeks [14, 15].

Strengths and limitations

The strength of our study is the characterisation of the UVB-source as well as the documentation of the UVB-exposure of the pigs during the study. Furthermore, a sensitive and specific analytical method capable of quantifying all four vitamin D metabolites was used for food and serum samples.

However, if the length of the exposure prior to slaughter had been extended beyond the 28 days it might lead to an additional increase in vitamin D as 50 days was needed to reach maximum serum vitamin D_3 concentration in mini-pigs exposed to UVB-light [10]. The UVB-tubes used had a maximum at 312 nm, however, the optimal wavelength for the production of vitamin D_3 in skin is 296 nm; at this wavelength approximately 10 times more vitamin D is produced compared to 312 nm [7]. If the spectrum of the used UVB-tubes was shifted so it had a maximum at 296 nm it would, however, only increase the erythemal effective irradiance with a factor of 3; therefore, in theory, the exposure time needed to obtain 1 SED would be 1/3, compared to at 312 nm, but the production of vitamin D_3 would increase approximately 3 times.

The variation caused by individual behavior could possibly be lowered by extending the exposure time, which could be implemented by increasing the height from lamp to pigs or using a UVB source with lower intensity.

Perspectives

It was demonstrated that it is possible to enhance the vitamin D content in pork by use of artificial UVB-exposure in indoor facilities for pigs, providing pork products with higher vitamin D content than has been shown in feeding trials. The obtained content by use of UVB-exposure is similar to the levels found in fish [43, 44]. Using UVB is a natural process for fortification with vitamin D that may be useful for marketing this pork given the public aversion to genetically modified organisms (GMO) products in Europe. Additionally, the vitamin D content is expected to be

unchanged during cooking as there is no significant difference between the vitamin D content in raw and cooked pork loin with rind [45].

The potential of our results (with only 28 days of exposure before slaughter) may be expressed based on the pig with the highest content of vitamin D; the total vitamin D activity in 100 g of minced meat with 10 % fat is calculated to be 3.0 µg and in a traditional Danish liver paté (35 % liver and 15 % lard) the activity is calculated to be 4.9 µg/100g; this is enough to be labelled 'high content of vitamin D' under current European legislation (Regulation (EC) No 1924/2006). However, the maximum production of vitamin D was probably not reached as we found a linear relationship between UVB dose and vitamin D content. Therefore, the UVB-lighting method described still calls for further investigation to realise its full potential to enhance vitamin D in pork.

Acknowledgements

We would like to acknowledge the staff at Rørrendegård (University of Copenhagen) for their commitment to take care of the pigs, Heidi Jahn and Tamaris Phipps (DTU Food) for their skillful performance of the analysis for vitamin D and fat, and Dennis Dan Corell (DTU Fotonik) for performing the UVB measurements.

Compliance with Ethical Standards

The Animal Experiments Inspectorate, part of the Danish Veterinary Food and Administration, judged that the trial did not need a governmental approval. No ethics committee was involved as the pigs lived similar to pigs at a farm except that they were exposed to UVB light similar to a daily exposure of approximately 5 to 20 minutes summer sunshine in Denmark. The project plan was assessed and approved by a veterinarian at the Department of Experimental Medicine at University of Copenhagen (Number P16-414). The animals were overseen by keepers and veterinarians during the trial.

Conflict of interest None.

References

1. Cashman KD, Dowling KG, Gonzalez-Gross M, et al (2016) Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr 103:1033–1044

2. Institute of Medicine (2011) Dietary Reference Intakes for Vitamin D and Calcium. The National Academies Press, Washington DC

3. Nordic Council of Ministers (2012) Nordic Nutrition Recommendations 2012 : Integrating nutrition and physical activity

4. Cashman KD, Kiely M (2016) Tackling inadequate vitamin D intakes within the population: fortification of dairy products with vitamin D may not be enough. Endocrine 51:38–46

5. O'Mahony L, Stepien M, Gibney MJ, et al (2011) The potential role of vitamin D enhanced foods in improving vitamin D status. Nutrients 3:1023–41

6. Black LJ, Seamans KM, Cashman KD, Kiely M (2012) An Updated Systematic Review and Meta-Analysis of the Efficacy of Vitamin D Food Fortification. J Nutr 142:1102–1108

7. Barnkob LL, Argyraki A, Petersen PM, Jakobsen J (2016) Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin. Food Chem 212:386–391

8. Kiely M, Black LJ (2012) Dietary strategies to maintain adequacy of circulating 25-Hydroxyvitamin D concentrations. Scand J Clin Lab 72:14–23

9. Holick MF (2007) Vitamin D Deficiency. N Engl J Med 357:266–281

10. Burild A, Frandsen HL, Poulsen M, Jakobsen J (2015) Tissue content of vitamin D3 and 25-hydroxy vitamin D3 in minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D3. Steroids 98:72–9

11. Jakobsen J, Jensen SK, Hymøller L, et al (2015) Short communication: Artificial ultraviolet B light exposure increases vitamin D levels in cow plasma and milk. J Dairy Sci 6492–6498

12. Schutkowski A, Krämer J, Kluge H, et al (2013) UVB exposure of farm animals: study on a food-based strategy to bridge the gap between current vitamin D intakes and dietary targets. PLoS One 8:e69418

13. Larson-Meyer DE, Ingold BC, Fensterseifer SR, et al (2017) Sun exposure in pigs increases the Vitamin D nutritional quality of pork. PLoS One 12:

14. Kolp E, Wilkens MR, Pendl W, et al (2017) Vitamin D metabolism in growing pigs: influence of UVB

irradiation and dietary vitamin D supply on calcium homeostasis, its regulation and bone metabolism. J Anim Physiol Anim Nutr (Berl) 101:79–94

15. Alexander BM, Ingold BC, Young JL, et al (2017) Sunlight exposure increases vitamin D sufficiency in growing pigs fed a diet formulated to exceed requirements. Domest Anim Endocrinol 59:37–43

16. Jakobsen J, Maribo H, Bysted A, et al (2007) 25-Hydroxyvitamin D3 affects vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin D. Br J Nutr 98:908–913

17. Burild A, Lauridsen C, Faqir N, et al (2016) Vitamin D3 and 25-hydroxyvitamin D3 in pork and their relationship to vitamin D status in pigs. J Nutr Sci 5:e3

18. Ovesen L, Brot C, Jakobsen J (2003) Food contents and biological activity of 25-hydroxyvitamin D: A vitamin D metabolite to be reckoned with? Ann Nutr Metab 47:107–113

19. Robertson CE (2003) McCance and Widdowson’s The Composition of Foods - Sixth Summary Edition. Nutr Bull 28:81–83

20. Tybirk P, Sloth NM, Kjeldsen N, Shooter L (2016) Nutrient requirement standards. 24th edition

21. Jakobsen J, Mikkelsen AÆ, Bysted A, Knuthsen P (2017) Næringsindhold i kødudskæringer - Danske grise. Søborg

22. Steenbock H, Hart EB, Jones JH (1924) Fat-soluble vitamins. XVIII. Sunlight in its relation to pork production on certain restricted rations. J Biol Chem 61:775–794

23. Steenbock H, Hart E, Hoppert C, Black A (1925) Fat-soluble vitamin. XXVI. The antirachitic property of milk and its increase by direct irradiation and by irradiation of the animal. J Biol Chem 66:441–449

24. Webb AR, Kline L, Holick MF (1988) Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab 67:373–8

25. International Commission on Illumination (1999) Erythema Reference Action Spectrum and Standard Erythema Dose. ISO/CIE Standard ISO 17166:1999

26. Burild A, Frandsen HL, Poulsen M, Jakobsen J (2014) Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. J Sep Sci 37:2659–63

27. Burild A, Frandsen HL, Jakobsen J (2014) Simultaneous quantification of vitamin D3, 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 in human serum by LC-MS/MS. Scand J Clin Lab Invest 74:418–423

28. International Standard Organization (2005) ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories. Int Stand 2005:1–36

29. Nordic Committee on Food Analysis. (1989) Fat. Determination according to SBR (Schmid–Bodynzki–Ratslaff) in meat and meat products. Method no. 131

30. R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing

31. RStudio Team (2016) RStudio: Integrated Development for R.

32. Bogh MKB, Schmedes A V., Philipsen PA, et al (2012) A small suberythemal ultraviolet B dose every second week is sufficient to maintain summer vitamin D levels: A randomized controlled trial. Br J Dermatol 166:430–433

33. Grigalavicius M, Moan J, Dahlback A, Juzeniene A (2015) Vitamin D and ultraviolet phototherapy in Caucasians. J Photochem Photobiol B Biol 147:69–74

34. Kühn J, Schutkowski A, Hirche F, et al (2015) Non-linear increase of vitamin D content in eggs from chicks treated with increasing exposure times of ultraviolet light. J Steroid Biochem Mol Biol 148:7–13 . doi: 10.1016/j.jsbmb.2014.10.015

35. Holick MF, MacLaughlin JA, Clark MB, et al (1980) Photosynthesis of Previtamin D3 in Human Skin And the Physiologic Consequences. Science (80-) 210:203–205

36. Jäpelt RB, Didion T, Smedsgaard J, Jakobsen J (2011) Seasonal Variation of Provitamin D 2 and Vitamin D 2 in Perennial Ryegrass (Lolium perenne L.). J Agric Food Chem 59:10907–10912

37. Wilson LR, Tripkovic L, Hart KH, Lanham-New SA (2016) Conference on “New technology in nutrition

research and practice" Postgraduate Symposium Vitamin D deficiency as a public health issue: using vitamin D 2 or vitamin D 3 in future fortification strategies. In: Procedings of the Nutrition Society. pp 11–14

38. Lehmann U, Hirche F, Stangl GI, et al (2013) Bioavailability of Vitamin D 2 and D 3 in Healthy Volunteers, a Randomized Placebo-Controlled Trial. J Clin Endocrinol Metab 98:4339–4345

39. Berg JM, Tymoczko JL, Stryer L (2006) Biochemistry, sixth. W.H. Freeman and Company

40. Clemens TL, Henderson SL, Adams JS, Holick MF (1982) Increased skin pigment reduces the capacity of the skin to synthesise vitamin D3. Lancet 319:74–76

41. Cashman KD, Kiely M, Kinsella M, et al (2013) Evaluation of Vitamin D Standardization Program protocols for standardizing serum 25-hydroxyvitamin D data: a case study of the program's potential for national nutrition and health surveys. Am J Clin Nutr 97:1235–1242

42. Jakobsen J, Bysted A, Andersen R, et al (2009) Vitamin D status assessed by a validated HPLC method: within and between variation in subjects supplemented with vitamin D 3. Scand J Clin Lab Invest 69:190–197

43. Padula D, Greenfield H, Cunningham J, et al (2016) Australian seafood compositional profiles: A pilot study. Vitamin D and mercury content. Food Chem 193:106–111

44. Jakobsen J, Smith C (2017) Farmed Salmon and Farmed Rainbow Trout - Excellent Sources of Vitamin D? Fish Aquac J 8:

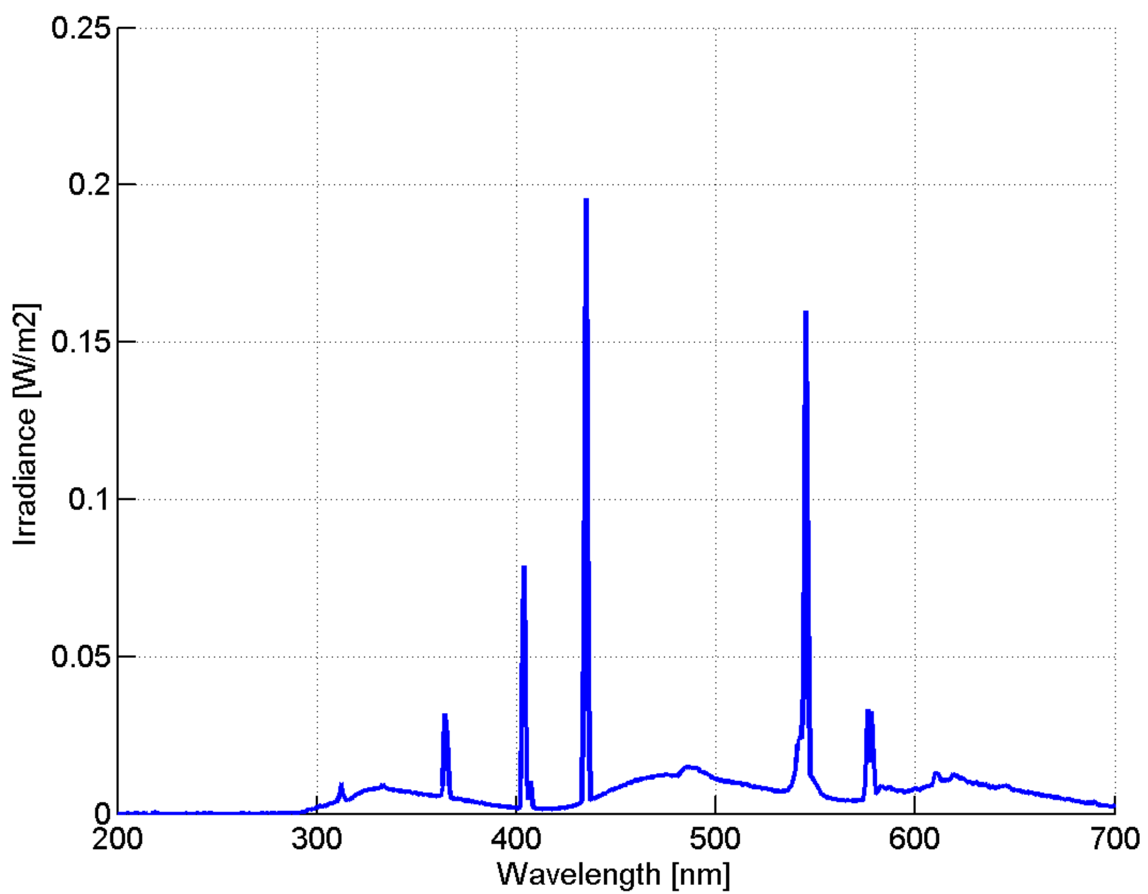
45. Clausen I, Jakobsen J, Leth T, Ovesen L (2003) Vitamin D3 and 25-hydroxyvitamin D3 in raw and cooked pork cuts. J Food Compos Anal 16:575–585

Online resource 1 to:

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

(in Eur Food Res Technol)

Corresponding author: Jette Jakobsen, National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark. jeja@food.dtu.dk



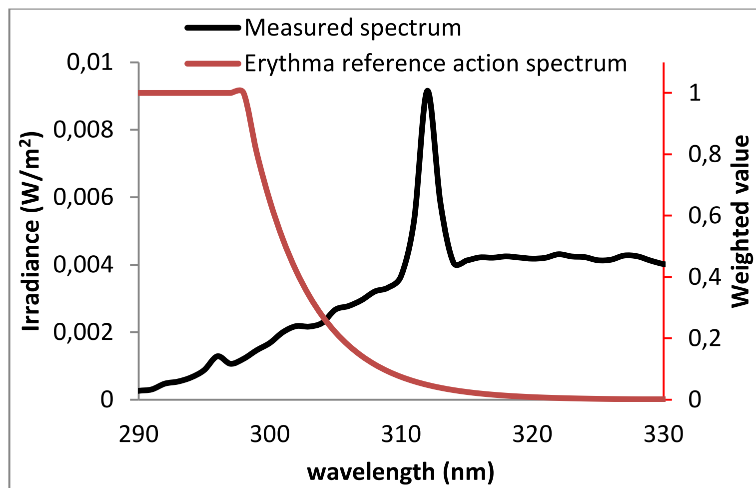
Online resource 1. The full spectrum of the UVB tube measured at a distance of 70 cm.

Online Resource 2 to:

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

(in Eur Food Res Technol)

Corresponding author: Jette Jakobsen, National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark. jeja@food.dtu.dk



Online Resource 2. The UVB part of the full spectrum from Figure S1 with the erythema reference action spectrum superimposed.

Online Resource 3 to:

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

(in Eur Food Res Technol)

Corresponding author: Jette Jakobsen, National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark. jeja@food.dtu.dk

Vitamin D analysis

The analytical method used on tissue samples, which included vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2, was developed by combining and optimizing different parts of two published methods for the quantification of vitamin D_3 and 25(OH)D_3 in serum and tissue [1, 2]. In short, the food samples were saponified over night at room temperature, clean-up by liquid-liquid extraction and solid phase extraction. For quantification on LC-MS/MS vitamin D metabolites were derivatised by 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). In serum samples the protein was precipitated and clean-up by solid phase extraction.

Chemicals and reagents

The following chemicals and standards were purchased from Sigma-Aldrich (Steinheim, Germany): Anhydrous acetonitrile, 99.8 %; Fluka Formic acid for MS; 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD); Ammonium formate; Cholecalciferol, analytical standard; Ergocalciferol, analytical standard; 25-hydroxyvitamin D_2, ≥ 98 % (HPLC; 25(OH)D_2); 25-hydroxyvitamin D_3 monohydrate, ≥ 99 % (HPLC; 25(OH)D_3). The three labeled internal standards used were: vitamin D_3-[26,26,26,27,27,27-d_6] and 25-hydroxyvitamin D_3-[26,26,26,27,27,27-d_6] from Chemaphor Inc. (Ottawa, Canada) and 25-hydroxyvitamin D_2-[25,26,27-$^{13}C_3$] obtained from IsoSciences (King of Prussia, PA). Ethyl acetate and 2-propanol, both HPLC-grade, were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). *n*-heptane (LC grade), methanol (LC-MS grade), ethanol (96%), Sodium ascorbate and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Milli-Q water was made in-house (18.2 MΩ, Millipore, Billerica, MA).

Stock solutions and calibration standards

Stock solutions of vitamin D_3 and D_2 were prepared in n-heptane; part of the solutions were evaporated and reconstituted in 95 % ethanol in order to determine the concentrations spectrophotometrically. Stock solutions of vitamin 25(OH)D_3 and 25(OH)D_2 were prepared in 96 % ethanol. The concentration of stock solutions were determined spectrophotometrically, at 265 nm with ε = 480 m^2/mol for vitamin D_3 [3], at 265 nm with ε = 475 m^2/mol for vitamin D_2 [3], at 264 nm with ε = 464 m^2/mol for 25(OH)D_3[4] and, at 246 nm with ε = 470 m^2/mol for 25(OH)D_2 [4]. Internal standard solution (IS), containing all three labeled standards, each with a concentration of 80 ng/mL, was prepared in 96 % ethanol. The calibration standards, dissolved in 96 % ethanol, were prepared from a mix of the four vitamers and the internal standard solution: the concentration of each of the four vitamers were 0.05, 0.1, 0.5, 1, 8, 10, 50, and 100 ng/mL, and the concentration of each of the labeled internal standards were 8 ng/mL at all levels. The calibration standards were derivatised with PTAD: first they were evaporated under nitrogen, then reconstituted in anhydrous acetonitrile containing 0.75 mg/mL PTAD, left to derivatise in the dark for five minutes and then 75 µL Milli-Q water was added to stop the reaction. Calibrations standards were included in all series of analyses.

Sample clean-up for food

The sample clean-up consisted of four steps: saponification, liquid-liquid extraction, solid phase extraction (SPE) and derivatisation. 1 gram of sample was added IS, 0.2 g sodium ascorbate, 9 mL ethanol, and 3 mL KOH (60%). 100 µL IS was added per 10 µg expected vitamin D_3 per 100 g sample, rounded down to the nearest 100 µL (i.e., if the expected content was between 11-20 µg/100g, 200 µL of IS was added). The sample tube was flushed with nitrogen and left to saponify overnight, at room temperature, placed in a Multi Reax (Heidolph, Essex, UK) at approximately 1700 rpm. 13

mL of Milli-Q water was added to the sample before it was extracted with 10 mL of 20 % ethyl acetate in n-heptane for 1 minute. The organic phase was transferred to a clean tube and the sample was re-extracted twice. The combined organic phase was washed with 20 mL of Milli-Q water. The organic phase was evaporated to dryness under vacuum in a centrifugal evaporator with a cold trap.

Two SPE methods were used, one for lean meat, subcutaneous fat and rind and another for liver. For lean meat, subcutaneous fat and rind the sample was immediately re-dissolved in 1000 µL 0.1 % formic acid in acetonitrile per 100 µL IS added (i.e. if 200 µL IS was added, 2000 µL would be used to re-dissolve). HybridSPE 30 mg/1 mL column (Supelco Analytical, Bellefonte, PA) placed in a vacuum manifold was used; the column was activated with 1 ml 0.1 % formic acid in acetonitrile without suction. 325 µL of the sample was loaded onto the column and eluted with 0.5 mL 0.1 % formic acid in acetonitrile; the columns were sucked dry using vacuum. For liver the sample was immediately re-dissolved in 4 mL 1 % 2-propanol in *n*-heptane. The sample was loaded onto a 6 mL silica 500 mg SPE cartridge (Isolute, IST, Hengoed, UK) activated with 4 mL of 0.5 % 2-propanol in *n*-heptane; washed twice with 4 mL of 0.5 % 2-propanol in *n*-heptane. The vitamers were eluted with 2 times 4 mL 6 % 2-propanol in *n*-heptane followed by 4 mL 10 % 2-propanol in *n*-heptane. The eluate was evaporated to dryness under nitrogen at 35 °C using a TurboVap® LV (Biotage, Uppsala, Sweden). The sample was immediately re-dissolved in 1000 µL 0.1 % formic acid in acetonitrile per 100 µL IS added (i.e. if 200 µL IS was added, 2000 µL would be used to re-dissolve).

The eluate from samples of lean meat, subcutaneous fat, and rind or 325 µL of the re-dissolved sample from liver was evaporated to dryness under nitrogen using a TurboVap® LV, and then re-dissolved in 250 µL 0.75 mg/mL PTAD in anhydrous acetonitrile. The sample was left to derivatise in the dark for five minutes and then 75 µL Milli-Q water was added to stop the reaction. The sample was centrifuged at 10,000 g for ten minutes before transferring it to a vial.

Sample clean-up for serum

The extraction procedure is describe in detailed elsewhere ([1, 2]. In short, 200 µL serum was added 300 µL acetonitrile, and 100 µL internal standard (80 ng/mL), followed by clean-up of the total volume on a HybridSPE. Next step was evaporation and derivatisation as described for food samples in the section above.

LC-MS/MS

Separation was performed on an Agilent 1200 Series HPLC; the column was an Ascentis Express C18 (2.1 mm X 10 cm, 2.7 µm particles) with an Ascentis Express C18 guard column (2.1 mm X 5 mm, 2.7 µm particles) from Supelco Analytical (Bellafonte, PA) thermostated at 50 °C. Mobile phase A was water:methanol (95:5) with 2.5 mM ammonium formate. Mobile phase B was methanol with 2.5 mM ammonium formate. The flow was 0.4 mL/min and the gradient was as follows: 0-1.5 min, 75 %B; 1.5-1.6 min, linear gradient to 85 %B; 1.6-3.5 min, 85 %B; 3.5-3.6 min, linear gradient to 98 %B; 3.6-5.9, 98 %B; 5.9-6 min, linear gradient to 75 %B; 6-10 min, 75 %B. The injection volume was 5 µL. The retention time was 2.6 min for 25(OH)D_3, 2.9 min for 25(OH)D_2 and 6.4 min for vitamin D_2 and D_3.

Quantification was performed on an Agilent 6470 Triple Quadrupole MS equipped with a Jet Stream ion source (Agilent Technologies, Santa Clara, CA) using positive multi reaction monitoring (MRM) mode. The instrument parameters used were: drying gas temperature, 300 °C; drying gas flow, 12 L/min; nebulizer pressure, 50 psi; capillary voltage, 3000 V; sheath gas temperature, 275 °C; sheat gas flow, 10 L/min; nozzle voltage, 500 V; cell accelerator voltage 3 V; delta EMV(+), 400 V; dwell time, 100 ms. Nitrogen was used as collision gas. Fragmentor and collision energy was optimized for each analyte. The $[M+H]^+$ ions of the derivatised analytes were used as precursor ions: vitamin D_3, 560.3 *m/z*; vitamin D_2, 572.3 *m/z*; vitamin D_3-d_6, 566.3 *m/z*; 25(OH)D_3, 576.3 *m/z*; 25(OH)D_3-d_6, 582.3*m/z*; 25(OH)D_2, 588.3 *m/z*; 25(OH)$^{13}C_3$, 591.3 *m/z*. Two product ions were used for each analyte, one for quantification (298.1 *m/z*) and one as qualifier (280.1 *m/z*). The *m/z* of the two product ions were the same for all the analytes.

Calibration curves and quality control

The precision and accuracy for all standards were < 5 % and 90-110 %, respectively. All standard curves were fitted as second order ln and weighted over the squared concentrations (1/x). The regression coefficients were > 0.999. Limit of quantification in food was 0.5 ng/g, except for 25OHD_2 in liver 1 ng/g, while in serum 0.2 ng/mL. Each series included an in-house reference sample of Control-pork fat, Control-pork liver or Control-serum. The precision of the method was < 10 % in food and < 5 % in serum. Furthermore the correctness of the method was checked by analyses of certified

reference materials (Milk powder, CRM421, IRMM, Geel, Belgium) and recovery of added vitamin D. The result was 151 ng vitamin D_3/g ± 2.4 % (n=8) which was within the certified values of 0.143 ± 0.008 mg/kg, while the recovery for vitamin D_3, vitamin D_2, 25(OH)D_3 and 25(OH)D_2 was 90-127 % (n=9). All of the analyses were conducted in a laboratory environment accredited to perform the analyses according to ISO17025 [5]

1. Burild A, Frandsen HL, Poulsen M, Jakobsen J (2014) Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. J Sep Sci 37:2659–63

2. Burild A, Frandsen HL, Jakobsen J (2014) Simultaneous quantification of vitamin D3, 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 in human serum by LC-MS/MS. Scand J Clin Lab Invest 74:418–423

3. Pharmaceutical Society of Great Britain. Dept. of Pharmaceutical Sciences (1979) The pharmaceutical codex - incorporating the British pharmaceutical codex, 11th ed. Pharmaceutical Press, London

4. Mattila PH, Piironen VI, Uusi-rauva EJ, Koivistoinen PE (1995) Contents of Cholecalciferol, Ergocalciferol, and Their 25-Hydroxylated Metabolites in Milk Products and Raw Meat and Liver As Determined by HPLC. JAgricFood Chem 43:2394–2399

5. International Standard Organization (2005) ISO/IEC 17025 General requirements for the competence of testing and calibration laboratories. Int Stand 2005:1–36

Online Resource 4 to:

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

(in Eur Food Res Technol)

Corresponding author: Jette Jakobsen, National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark. jeja@food.dtu.dk

Online Resource 4. Association between vitamin D_3 status and content of vitamin D_3 in other parts of the pig.

	Pearson's R	
Part of the pig	Vitamin D_3	25(OH)D_3
Rind	0.92	0.97
Subcutaneous fat	0.95	0.95
Lean meat	0.96	0.97
Liver	0.98	0.98
Serum	0.96	-

Online resource 5 to:

Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities

(in Eur Food Res Technol)

Corresponding author: Jette Jakobsen, National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark. jeja@food.dtu.dk

Figure 1. Vitamin D_3 content as a function of UVB dose.

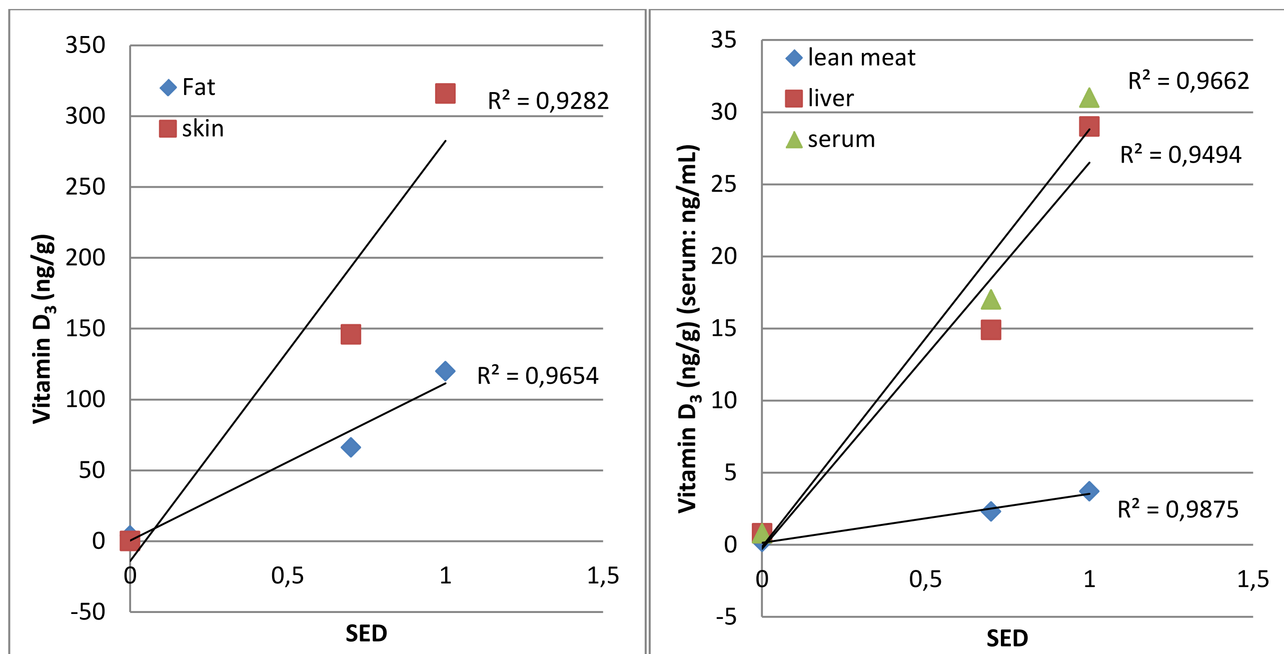
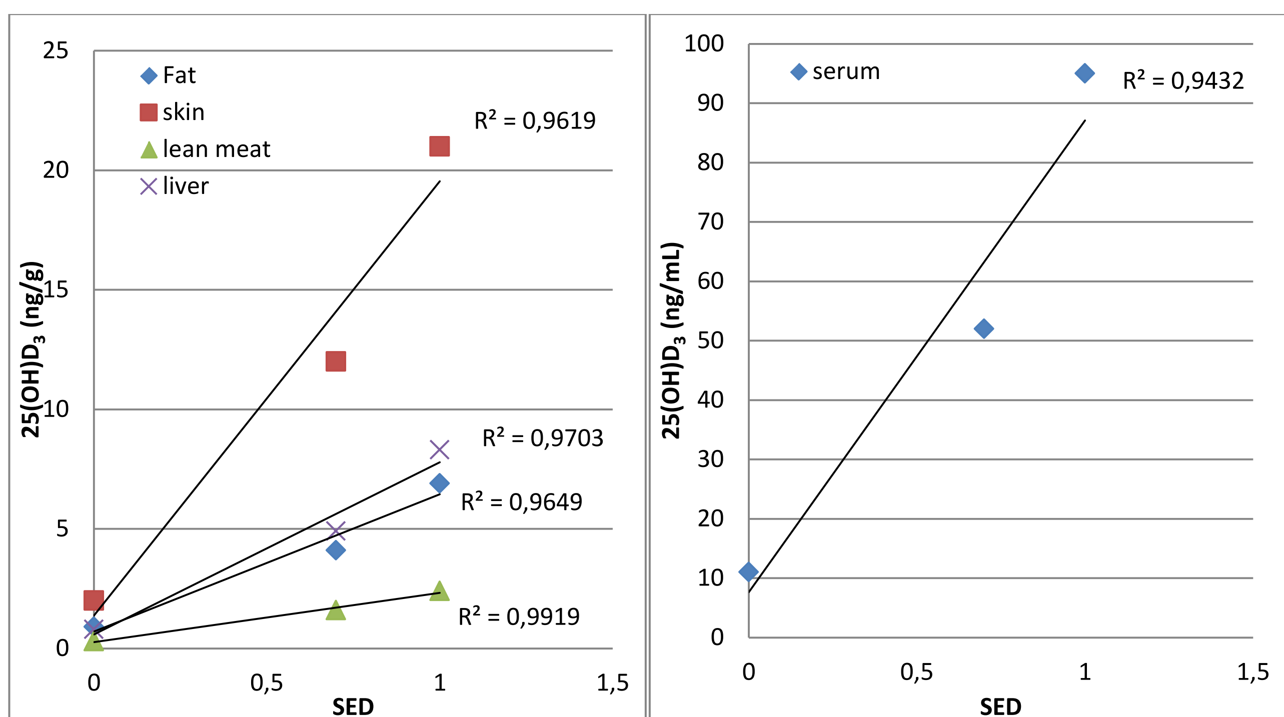


Figure 2. 25-hydroxy vitamin D_3 content as a function of UVB dose.



Paper III

Naturally enhanced eggs as a source of vitamin D: A review

Line Lundbæk Barnkob[a], Aikaterini Argyraki[b] and Jette Jakobsen[a]*

[a] National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark

[b] Technical University of Denmark, Department of Photonics Engineering, DTU Risø campus, Frederiksborgvej 399, 4000 Roskilde, Denmark

* Corresponding author

E-mail: jeja@food.dtu.dk (J. Jakobsen)

Abstract

Background: It is estimated that on annual basis 40 % of the European population is either vitamin D insufficient/deficient. A way to increase the vitamin D intake is to fortify a broader range of foods or by increasing the natural vitamin D content in food sources that already contain vitamin D. Eggs is once again considered part of a healthy varied diet and eggs contain a wide range of micro nutrients including vitamin D.

Scope and Approach: Review of production methods to naturally enhance eggs with vitamin D, and discussion of the perspectives of vitamin D enhanced eggs as part of the strategy to increase the dietary intake of vitamin D.

Key Findings and Conclusions: There are three ways to naturally enhance the vitamin D content in eggs: feeding more vitamin D_3/25(OH)D_3 to the hens, exposing the hens to UVB and exposing liquid egg products to UVB. Naturally enhanced eggs can contribute to increased vitamin D intake. An inter-trial linear relationship between vitamin D_3 in feed and vitamin D_3 in eggs was found. Within the linear range a maximum of 20 µg/100 g yolk was obtained with feed contain 617.5 µg/kg feed. Feed can provide higher levels of vitamin D in eggs than UVB exposure of the hens. However, the European maximum for vitamin D in feed for layers at 80 µg/kg limits the beneficial effect. Vitamin D content in liquid egg products can be tailored by adjusting the UVB dose, however further research is needed.

Keywords

Vitamin D_3; fortification;25-hydroxyvitamin D_3; hens; UVB; feed

Highlights

- Three methods to produce eggs naturally enhanced with vitamin D was reviewed
- There is a linear inter-trial relationship between vitamin D_3 in feed and vitamin D_3 content in egg
- Feed is superior to UVB irradiation in regards to vitamin D enhancement in eggs
- Egg yolk can be vitamin D enhanced by direct exposure to UVB radiation
- Naturally enhanced eggs can contribute to increased vitamin D intake in the general population

1. Introduction

It is estimated that on annual basis 40 % of the European population is either vitamin D insufficient (< 50 nmol/L total serum 25-hydroxyvitamin D (25(OH)D) or deficient (< 30 nmol/L) (Cashman et al., 2016). Although there is dispute about where to set the limit of insufficiency and deficiency, there is agreement that the vitamin D status (total serum 25(OH)D) of the general population has to be increased (Holick, 2017).

Vitamin D can be obtained either through food or sun exposure, where sun exposure is the major contributor to vitamin D in humans. 7-dehydrocholesterol (7-DHC) in the skin is converted to previtamin D_3 when the skin is exposed to wavelengths between 290 and 315 nm (ultraviolet B, UVB) and previtamin D_3 is then converted to vitamin D_3 by thermal isomerization at body temperature (Holick et al., 1980; MacLaughlin, Anderson, & Holick, 1982); vitamin D_3 is transported to the liver where it is converted to 25-hydroxivitamin D_3 (25(OH)D_3) (Christakos, Dhawan, Verstuyf, Verlinden, & Carmeliet, 2016). However, from October through March at 52 °N no cutaneous vitamin D is produced as most of the solar photons below 315 nm is attenuated by the longer travel through the ozone layer during the so-called vitamin D winter (Webb, 2006; Webb, DeCosta, & Holick, 1989; Webb, Kline, & Holick, 1988). The duration of the vitamin D winter decreases with decreasing latitude and according to UVB data it is suggested that the duration is: 5 months in UK, Ireland and the Netherlands; 6 months in Denmark; 8 months in the north of Norway; while it is 2 to months in Greece and 0 in Crete (O'Neill et al., 2016).

Vitamin D exists in two forms: vitamin D_2 and vitamin D_3. Although there are multiple metabolites of vitamin D, only vitamin D and its major metabolite 25(OH)D is considered when determining the total vitamin D content in food (Ovesen, Brot, & Jakobsen, 2003). Vitamin D_3 and 25(OH)D_3 is found in food of animal origin, such as fish, meat, offal, eggs, milk and dairy products (Ovesen et al., 2003) Vitamin D_2 is found in yeast, mushrooms, and fortified milk and dairy products (Keegan, Lu, Bogusz, Williams, & Holick, 2013).

The recommended intake of vitamin D is between 10 and 20 µg/day (Institute of Medicine, 2011; Nordic Council of Ministers, 2012), however the estimated intake is 3 to 7 µg/day (Cashman & Kiely, 2016). In some countries with mandatory fortification programmes the intake of vitamin D is still suboptimal (Lehtonen-Veromaa et al., 2008; Whiting, Green, & Calvo, 2007). Since 2003 Finland have had a voluntary fortification programme which includes milk and fat spread with the effect that the adult population who have a diet based on nutritional recommendations have a sufficient vitamin D status, while the status in adolescent girls is still insufficient (Jääskeläinen et al., 2017; Lehtonen-Veromaa et al., 2008)
A way to increase the vitamin D intake is to fortify a broader range of foods or by increasing the natural vitamin D content in food sources that already contain vitamin D (Barnkob, Argyraki, Petersen, & Jakobsen, 2016; Black, Seamans, Cashman, & Kiely, 2012; Cashman & Kiely, 2016; Kiely & Black, 2012; O'Mahony, Stepien, Gibney, Nugent, & Brennan, 2011). Foods with natural increased vitamin D content could in some cases be more broadly accepted by consumers (Cashman, 2015).

For many years eggs have had a bad reputation due to a high content of cholesterol, however as reviewed by Gray (2018), eggs were wrongfully accused and is once again considered to be part of a healthy varied diet as they contribute with a range of micro nutrients, including vitamin D, and high quality, easily digestible proteins; and compared to other food sources eggs are easily prepared, they are relatively cheap and they are included in the diet of the majority of the population (Gray, 2018; Mejborn, Jacobsen, & Trolle, 2011; Pedersen et al., 2015).

The natural content of vitamin D in eggs can be increased by three methods, of which two have gained the most attention: adding more vitamin D to the feed of the hens; and exposing the hens to UVB light. The third method is to expose egg yolk directly to UVB. We will review the literature on these three methods and discuss their future perspectives.

2. Method

An extensive literature search was performed in January 2019. The search machine DTU Findit (DTU Library, n.d.) was used with the following search phrase: vitamin D OR eggs OR ultraviolet OR content OR yolk. Reference lists from relevant articles found in the search as well as the cited by function in Google were used to find additional references.

In order to make different reported results comparable all results have been recalculated to µg/100 g egg for vitamin D_3 and 25(OH)D_3; and µg/kg for vitamin D_3 and 25(OH)D_3 in feed. Some authors report the results per gram egg yolk, in these cases the data has been recalculated under the assumption that a whole egg consists of 33 % yolk (Mattila, Lehikoinen, Kiiskinen, & Piironen, 1999; Mattila, Valkonen, & Valaja, 2011). Others have reported results per gram dry matter of egg yolk, it is assumed that an egg yolk contain 7 g of dry matter (DTU Food, 2018; Kühn et al., 2015; Schutkowski et al., 2013) based on an average egg yolk weight of 15 g. It is assumed that vitamin D is only present in the yolk.

To make UVB doses comparable all reported values have been recalculated to J/m^2.

2.1. Limitations

This review will primarily focus on the vitamin D content, if interested in how vitamin D and its metabolites affect performance and egg quality we refer to a recent review from Świątkiewicz, Arczewska-Wlosek, Bederska-Lojewska, & Józefiak (2017).

Vitamin D_2 is not as effectively transferred to yolk as vitamin D_3 (Francis G. McDonald & Massengale, 1932), and only vitamin D_3 and 25(OH)D_3 is approved as vitamin D additives in feed for layers; therefore studies performed with vitamin D_2 is not included except for Kawazoe, Yuasa, Yamazaki, & Ando (1994) who studied the relation between feed intake and vitamin D content in eggs which have not been studied using vitamin D_3.

A lot of studies were published in the 1920'ties and 1930'ties regarding how to increase the vitamin D content in eggs. It was shown that the content of vitamin D in egg yolk could be increased by adding vitamin D to the feed of laying hens (Bethke, Kennard, & Sassaman, 1927; Branion, Drake, & Tisdall, 1935; DeVaney, Munsell, & Titus, 1933; Guerrant, Kohler, Hunter, & Murphy, 1935) or by irradiating non-supplemented hens from above (Farrell, 1924; Hart et al., 1925; Hendricks, 1931; Hughes & Payne, 1924; Hughes, Payne, Titus, & Moore, 1925; Maughan & Maughan, 1933). However, when Carson & Beall (1955) supplemented hens with approximately 29 µg vitamin D/kg feed and exposed them to UVB from above, no increase of vitamin D in the eggs were observed. However, the vitamin D content in these studies of older date was determined by hatchability of eggs or by animal assays where the total vitamin D activity is determined. Only studies that measure the individual content of vitamin D_3 and/or 25(OH)D_3 have been included.
Two studies were excluded as they also included increased content of vitamin K in the feed (Park, Namkung, Ahn, & Paik, 2005; Zang et al., 2011). At high concentration vitamin K inhibits the uptake of vitamin D (Reboul, 2015) and as both vitamin D and vitamin K are involved in bone health (Torbergsen et al., 2015) it cannot be ruled out that they also interact at higher levels in vivo.

3. Bioavailability of vitamin D from eggs

As mentioned in the introduction eggs are an optimal vehicle for vitamin D in regards to its nutritional qualities and wide use. A recent review found no difference in the bioavailability of vitamin D_3 from fortified food compared to supplements (Borel, Caillaud, & Cano, 2015). Investigation of the bioavailability of vitamin D_3 and 25(OH)D_3 from natural food is limited, but no difference were shown for vitamin D_3 from cod liver oil and vitamin D_3 in multivitamin tables to increase vitamin D status (Holvik, Madar, Meyer, Lofthus, & Stene, 2007). Moreover, it has been shown than intake of vitamin D enhanced eggs is associated with increased vitamin D status (Hayes et al., 2016).

4. Cooking loss of vitamin D in eggs

Two studies regarding the loss of vitamin D during cooking of eggs have been published (Jakobsen & Knuthsen, 2014; Mattila, Ronkainen, Lehikoinen, & Piironen, 1999). Vitamin D and 25(OH)D in eggs will be lost, to the same extent, during household cooking: A hardboiled egg (10 minutes of cooking) will lose around 10 % of both vitamin D and 25(OH)D, scrambling an egg for 3 minutes gives less than 20 % loss of both, while during baking for 40 minutes around 60 % of both is lost (Jakobsen & Knuthsen, 2014; Mattila, Ronkainen, et al., 1999).

5. European legislation regarding vitamin D in feed for laying hens

The legal limit for adding vitamin D_3, 25(OH)D_3 or a combination of the two to feed is 80 µg/kg for laying hens in Europe (Commission Regulation (EU) 2017/1492 Commission Regulation (EC) No 887/2009); according to Commission Regulation (EU) 2017/1492 this level does not have adverse effects on animal health, human health or the environment based on scientific opinions from the European Food Safety Authority (EFSA). The reasoning behind EFSAs conclusion regarding animal safety was that this level has been used for over a decade without any reported intolerances (EFSA, 2014); they were however unable to draw any final conclusion as their answer was solely based on data from the National Research Council (NRC) from 1987. In regard to human health, they conclude that as there has been no change in the level of vitamin D in feed during the last decade it is safe to continue with the same level (EFSA, 2014). The fact that the human intake of vitamin D is below the recommended was not taken into consideration.

6. Vitamin D content in commercial feed for laying hens

In 2014 one of the major producers of feed for laying hens in Denmark used 75 µg vitamin D_3 per kg feed (personal communication with Danish Agro). A survey from 1996 conducted in USA showed that the average content of vitamin D_3 in feed for laying hens was 61.5 µg/kg feed (BASF, 1998), while in China the average level is 60 µg/kg feed (Zang et al., 2011).

How common the use of 25(OH)D_3 in feed has become since it was legalized in 2009 in Europe is unknown but Mattila et al. (2011) concluded from their investigations of commercial eggs that egg producers in Finland so far continued to use vitamin D_3 as the only vitamin D supplement, however in 2011 one producer in Denmark launched an egg where 25(OH)D_3 was used in the feed (Hedegaard, n.d.) and Liu, Greenfield, & Fraser (2014) analysed eggs from an Australian producer known to use a combination of 25(OH)D_3 and vitamin D_3.

7. Vitamin D content in commercial eggs

The content of vitamin D_3 and 25(OH)D_3 in commercial eggs reported by various sources since 1982 is displayed in Table 1. On average the content of vitamin D_3 and 25(OH)D_3 in commercial eggs is 1.5 µg/100g and 0.5 µg/100g, respectively.

Table 1. Average content of vitamin D_3 and 25(OH)D_3 in commercial whole eggs

Origin	Vitamin D_3 (µg/100g)	25(OH)D_3 (µg/100g)	Reference
England	1.6	-	(Jackson, Shelton, & Frier, 1982)[b]
England	1.1	-	(Sivell, Wenlock, & Jackson, 1982)
Japan	1.3	-	(Takeuchi, Okano, Teraoka, Murakami, & Kobayashi, 1984)
Finland	1.7	0.32	(Mattila et al., 1992; Mattila, Piironen, Uusi-rauva, & Koivistoinen, 1993)
Canada	0.92	0.36	(Bilodeau et al., 2011)[a]
Finland	1.4	0.38	(Mattila et al., 2011)
USA	2.0	0.65	(Exler, Phillips, Patterson, & Holden, 2013)[a]
Russia	2.2	-	(Chirkin, Karpov, Selemenev, & Shumskiy, 2013)
Australia	0.83	0.92	(Liu et al., 2014)
England	2.5	0.13	(Publich Health England, 2015)[a]
Ireland	1.1	1.0	(Hayes et al., 2016)
England	1.7	0.5	(Guo, Kliem, Lovegrove, & Givens, 2017)[b]
Australia	0.95	0.9	(Dunlop et al., 2017) [b]
Denmark	1.9	0.71	(DTU Food, 2018)[a,b]
Average (SD)	**1.5 (±0.5)**	**0.5 (±0.3)**	

SD = Standard deviation

\- = not analysed

[a] Food composition table or data produced for a food composition table

[b] calculated average of results from free range, indoor and organic hens

8. Effect of enhancing vitamin D content through feed

Vitamin D in feed can be given either as vitamin D_3, 25(OH)D_3 or a combination of the two. Hens have a binding protein for vitamin D_3 in the plasma that forms a complex that is actively transported into yolk; however this binding protein also has a small affinity for 25(OH)D_3 and when 25(OH)D_3 is present in high concentrations it can displace vitamin D_3 and thereby be actively transported into yolk (Fraser & Emtage, 1976). When feeding vitamin D_3 the content of both vitamin D_3 and 25(OH)D_3 in the egg will increase, however using 25(OH)D_3 alone will not increase the vitamin D_3 content of the egg (Browning & Cowieson, 2014); and when fed a combination the displacement of vitamin D_3 by 25(OH)D_3 mentioned above is assumed to be minimal. Therefore studies that use a combination have been included in assessing the impact feeding vitamin D_3 has on the vitamin D_3 content in eggs.

When starting a new feeding regime it will take some time before the vitamin D_3 content in the eggs reach a stable plateau (equilibration). The equilibration time is reported to be between 8 days and 3 weeks and it is independent of the vitamin D_3 content of the feed; and when an equilibrium is reached the content stays relatively stable (Mattila et al., 2003; Park et al., 2005; Yao, Wang, Persia, Horst, & Higgins, 2013). The duration of the studies feeding vitamin D_3 was between 2 weeks and 48 weeks while for 25(OH)D_3 it was between 4 and 9 weeks.

The age of the hens varies between trials, but Mattila et al. (2003) found that age does not have an effect on the transfer of vitamin D_3 from feed to the egg and it is assumed that this holds true for 25(OH)D_3 as well. A variation of commercial breeds, both white and brown, has been used.

It should be noted that the reported values are averages and that the content in the individual eggs will vary as the feed intake per hen varies. Kawazoe et al. (1994) found an average feed intake of 117 g ± 7.6 g (standard deviation, SD) and they showed a linear relationship between vitamin D content in the egg and the feed intake; within a group the vitamin D content varied between 7-11 μg/100 g and the coefficient of variance (CV) of the mean was 15 %[1]. Kawazoe et al. (1994) used irradiated shitake and thereby D_2, but it is assumed that relation will be the same for vitamin D_3 and 25(OH)D_3. The CV% of vitamin D_3 and 25(OH)D_3 determined in egg yolk, fed a combination of the two, were comparable with an average CV of 24 % (Browning & Cowieson, 2014). Genetic differences between birds are most likely also a contributor to the observed variance (Browning & Cowieson, 2014).

8.1. Effect of using vitamin D_3 as feed additive

We found eight studies where hens have been fed vitamin D_3 at levels between 26.6 μg/kg and 2555 μg/kg (Browning & Cowieson, 2014; Hayes et al., 2016; Kawazoe, Yuasa, Noguchi, Yamazaki, & Ando, 1996; Mattila et al., 2003; Mattila, Valaja, Rossow, Venäläinen, & Tupasela, 2004; Mattila, Lehikoinen, et al., 1999; Plaimast, Kijparkorn, & Ittitanawong, 2015; Yao et al., 2013).

The transfer efficiency (= [vitamin D_3 in yolk • yolk weight • egg production per hen per day]/[vitamin D_3 in feed • feed intake per hen per day • 100]) of vitamin D_3 from diet to egg was determined by Kawazoe et al. (1996) and Yao et al. (2013). The data from the two studies have shown that the transfer efficiency increases when the content in feed is increased: it is 7-8 % when using 55 μg/kg feed; 11-14 % when using 242.5-617.5 μg/kg; and 41-47 % when using 2555 μg/kg. Yao et al. (2013) also calculated transfer efficiencies using data from other studies where similar levels of supplementation was used and the results were within the ranges given above.

In long term trials (≥ 24 weeks) no negative health effects of feeding 300 to 2555 μg vitamin D_3 /kg feed have been observed (Mattila et al., 2003, 2004; Persia, Higgins, Wang, Trample, & Bobeck, 2013). Reduced feed intake, egg weight, shell quality and fertility have been reported for laying hens fed with 5000 μg/kg (Ameenuddin, 1986).

The vitamin D_3 content in eggs as a function of the content vitamin D_3 in feed is displayed in Figure 1.

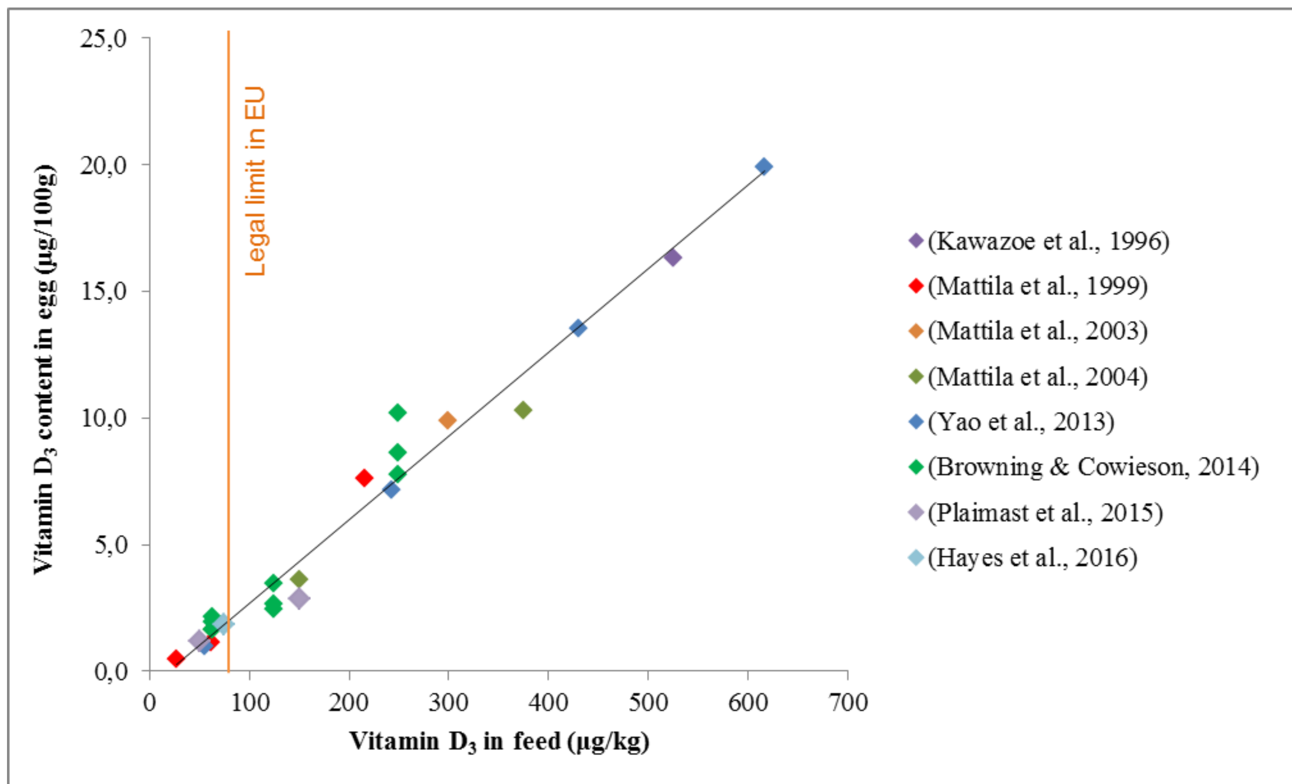
There is a linear relationship between the vitamin D_3 content in the feed and the content of vitamin D_3 in the egg with a Pearson correlation coefficient (Pearson's r) of 0.987 and a p-value of $6 \cdot 10^{-19}$ (F-test) in spite of the differences in the design of the studies. The equation for the linear regression is:

$$\text{Vitamin } D_3 \text{ in eggs } (\mu g/100g) = 0.033 \cdot \text{vitamin } D_3 \text{ in feed } (\mu g/kg) - 0.58$$

[1] Approximately values as they are calculated from data points in Figure 6 in the paper.

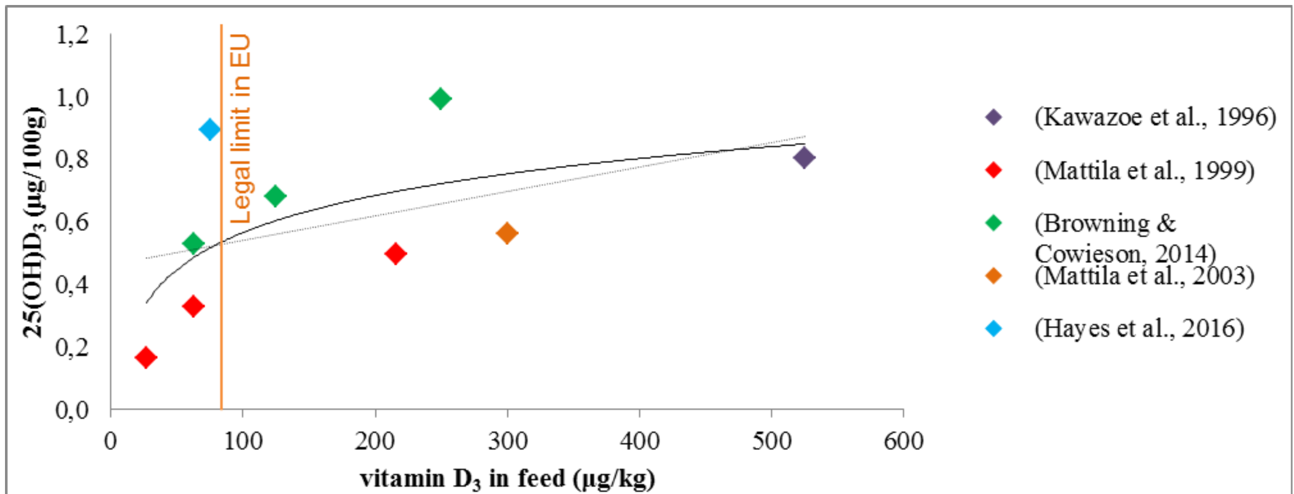
The linear range covers up to 617.5 µg/kg feed and the highest vitamin D content in eggs is 40 times higher than the lowest. Yao et al. (2013) fed 2555 µg/kg feed to one of their treatment groups and the resulting vitamin D_3 content was 287 µg/100 g; this result is not included in Figure 1 as it is outside the linear range; the expected content from the linear regression would be 84 µg/100 g. This fits with the observation that the transfer efficiency increases with increased content of vitamin D_3 in the feed (Kawazoe et al., 1996; Yao et al., 2013).

Figure 1. Vitamin D_3 content in eggs as a function of vitamin D_3 content in feed



The 25(OH)D_3 content in eggs as a function of vitamin D_3 in feed is better described by a logarithmic equation (black line in Figure 2) with a Pearson's r of 0.6 compared to 0.47 for the linear relationship (dotted line in Figure 2). However, the data is limited and the p-value for the F-test of the regression of the log-transformed data is 0.08. The only conclusion that can be draw is that 25(OH)D_3 content in eggs will increase with increased supplementation of vitamin D_3 but to a lesser degree than what is observed for the vitamin D_3 content in eggs.

Figure 2. 25(OH)D_3 content in eggs as a function of vitamin D_3 content in feed. Full black line is exponential regression while dotted line shows linear regression of the data.

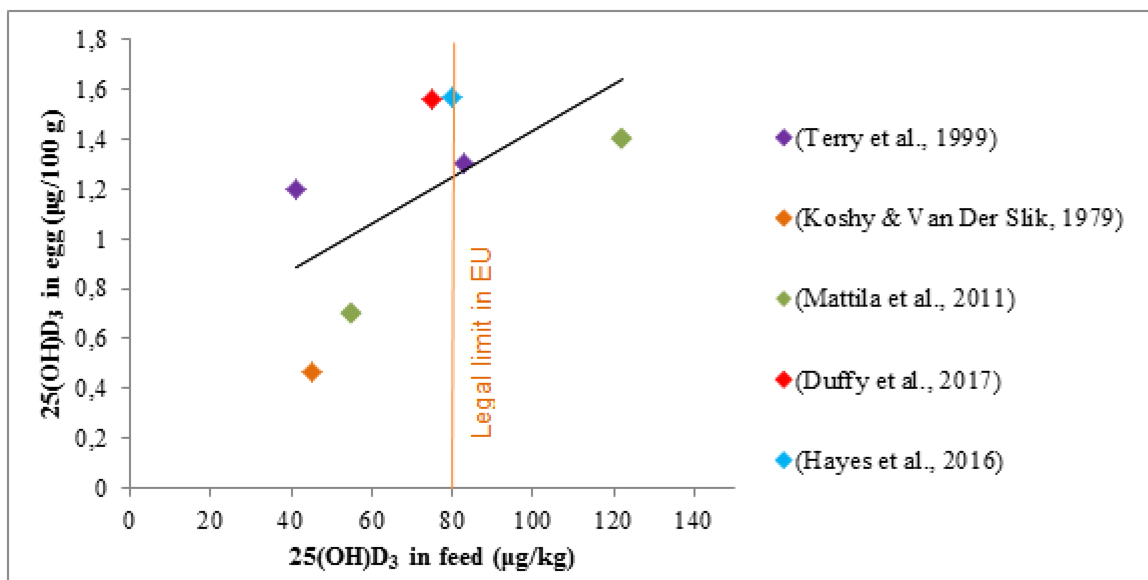


8.2. Effect of using 25(OH)D_3 as feed additive

We have found six studies that report on the vitamin D content of eggs after hens have been fed 25(OH)D_3 alone or in combination with vitamin D_3 (Browning & Cowieson, 2014; Duffy et al., 2017; EFSA, 2005; Hayes et al., 2016; Koshy & Van Der Slik, 1979; Mattila et al., 2011).

Figure 3 shows the 25(OH)D_3 content in eggs from hens that have been fed solely 25(OH)D_3. From Figure 3 it is seen that only two studies have used at least two levels and that Mattila et al. (2011) is the only of the two that shows a significant increase; however, with a Pearson's r of 0.6 there is not the same linear inter-trial tendency as shown for vitamin D_3, but the limited amount of data should be taken into consideration. The dose-response is moderate compared to what is observed for vitamin D_3; the slope of the linear regression is 0.0093 which is approximately a factor 3 lower than the vitamin D_3 slope (see section 8.1).

Figure 3. $25(OH)D_3$ content in eggs as a function of $25(OH)D_3$ content in feed.

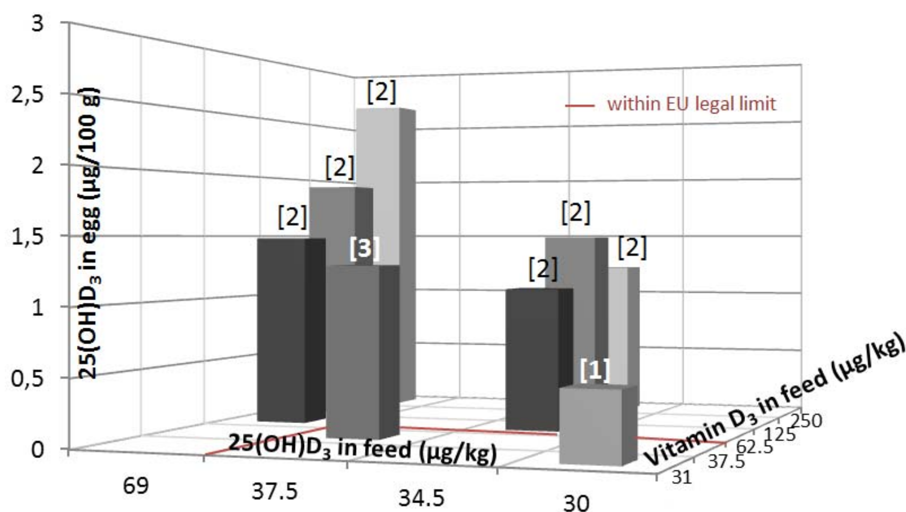


Only two studies used doses above the legal limit of 80 µg/kg. Terry, Lanenga, McNaughton, & Stark (1999) supplemented laying hens with 41, 83, 413 and 825 µg $25(OH)D_3$ per kg feed for 224 days, however eggs were analysed for vitamin D content after 112 days. No mortality was observed at any level. Based on decreased production parameters 825 µg were toxic while 413 µg $25(OH)D_3$ per kg feed were just above the margin of the no effect level and for this reason the results of these two treatments are left out of Figure 3. Eggs from hens fed 413 µg/kg had a $25(OH)D_3$ content of 2.4 µg/100 g (Terry et al., 1999), therefore this could be considered to be the maximum obtainable level when using $25(OH)D_3$ as the only vitamin source. Mattila et al. (2011) fed hens with 122 µg $25(OH)D_3$/kg feed during 6 weeks without observing any negative effects on the egg shell quality.

Based on the results presented in Terry et al. (1999) 80 µg/kg was set as the safe level in the scientific opinion from EFSA regarding the use of $25(OH)D_3$ in feed (EFSA, 2005).

Three studies used a combination of $25(OH)D_3$ and vitamin D_3 (Browning & Cowieson, 2014; Duffy et al., 2017; Mattila et al., 2011); the results are displayed in Figure 4. From the results of Browning & Cowieson (2014) ([2] in Figure 4) it can be seen that increasing the $25(OH)D_3$ supplementation while keeping the vitamin D_3 supplementation unchanged will give increased $25(OH)D_3$ content in eggs. Feeding a combination of 250 µg vitamin D_3 and 69 µg $25(OH)D_3$ results in a $25(OH)D_3$ content of 2.7 µg /100 g in the eggs; this exceeds the maximum that can be obtained by feeding $25(OH)D_3$ alone.

Figure 4. $25(OH)D_3$ content in eggs from hens fed $25(OH)D_3$ in combination with vitamin D_3. [1] Mattila et al. (2011); [2] Browning & Cowieson (2014); [3] Duffy et al. (2017).



9. Enhancement using artificial UVB

9.1. Location and content of 7-DHC in hens

The legs and feet of hens have the highest content of 7-DHC when compared to the feathered part of the hens and the comb. Koch & Koch (1941) found that skin from the legs and feet of chicken had approximately 8 times as much 7-DHC compared to the body. Tian, Chen, Lu, Shau, & Holick (1994) measured the content of 7-DHC in various parts of the chicken skin. They found that the 7-DHC content in skin on the legs and feet were 29 and 23 times higher than the content in the back skin. After irradiation with 5000 J/m^2 no pre-vitamin D_3 was detected in the back skin (Tian et al., 1994). Schutkowski et al. (2013) found that the 7-DHC concentration in the un-feathered legs was 190 times higher than the concentration in the comb; the lowest concentration was found in the feathered parts of the hen. Edwards (2003) irradiated young chickens with UVB and looked at growth and bone development; he found that exposure from below was more than twice as effective as from above.

Uva, Mandich, & Vallarino (1983) found that vitamin D_3 concentration was 3 times higher in the uropygial gland than in the unfeathered skin from the legs and concluded that this is the major site for production of vitamin D_3 (from 7-DHC). They also found vitamin D_3 in the extracts of feathers, the origin was hypothesized to be from the gland and that the oil was spread in the act of preening. Tian et al. (1994) found 7-DHC in lipid extracts from feathers; this could originate from the uropygial gland as Uva et al. (1983) hypothesized as the major production site of 7-DHC.

9.2. Effect of exposing hens to UVB from above

It has been shown possible to increase vitamin D in eggs when irradiating hens from above, if the hens do not receive vitamin D through the feed (Chiang, Hwang, & Holick, 1996, 1997). However, Lietzow et al. (2012) irradiated layers, with UVB light in the range of 280-310 nm for 4 weeks from above, with a dose of 540 J/m^2/day. Both non-supplemented hens and hens supplemented with 75 µg vitamin D_3/kg feed were exposed. They found no effect of UVB exposure on the content of vitamin D in eggs. However, as no increase was observed in the non-supplemented group, as would have been expected from the trials of Chiang et al. (1996, 1997) and older findings, the dose might have been too low. We have repeated the trial of Lietzow et al. (2012) with a dose of 547 J/m^2/day using UVB light emitting diodes (LED) with a central wavelength of 307 ± 2 nm (standard deviation) which should be more optimal for vitamin D_3 production (Barnkob et al., 2016); the outcome was however the same (Argyraki, 2017). We also tried with a dose of 4000 J/m^2/day, however, the treatment was stopped after 7 days as changes of the comb was observed and interpreted as erythema; in retrospect the changes could be harmless as free range hens have more red and stiff comb than hens secluded from sunlight. Eggs collected before UVB exposure had a content of 1.1 µg/100 g while eggs collected after 7 days of exposure, where the treatment was stopped, had a vitamin D_3 content of 1.6 µg/100 g; equilibrium was probably not reached as the content 5 days after ceased exposure was 1.7 µg/100 g (Argyraki, 2017).

9.3. Effect of exposing the legs of hens to UVB

Schutkowski et al. (2013) exposed the legs of hens, supplemented with 75 µg vitamin D_3/kg feed, to a dose of 8208 J/m^2/day for 4 weeks; the reported content of vitamin D_3 and 25(OH)D_3 is 4,9 µg/100 g and 0,57 µg/100 g respectively. Kühn et al. (2015) irradiated the legs of hens to 7 different doses of UVB between 0-13680 J/m^2/day during 4 weeks and showed that vitamin D_3 in the eggs increased non-linearly with dose and at the highest dose the content was 4.5 µg/100 g and it was almost at equilibrium. 25(OH)D_3 content of eggs also increased non-linearly but did not increase beyond a dose of 5472 J/m^2/day where equilibrium was reached with a content of 0.6 µg/100g.

10. Vitamin D enhancement of liquid egg products by direct exposure to UVB

Egg yolk contain around 140 µg 7-DHC per 100 g (Kühn, Schutkowski, Kluge, Hirche, & Stangl, 2014); therefore there is a potential that egg yolk can be enhanced with vitamin D by exposing it directly to UVB. In a small pilot study, described in Argyraki (2017), we exposed egg yolk and whole egg mix to UVB light using the UVB-LED with a central wavelength of 296 nm described in Barnkob et al. (2016). Layers of 3-4 mm liquid egg were placed in small weigh boats and then exposed to a dose of 3000 J/m^2; the exposure time was approximately 3.5 min. In whole egg the vitamin D_3 content increased from 1.1 to 4.3 µg/100 g and in

egg yolk the content increased from 5.2 to 18.9 µg/100 g; close to a factor of 4 in increased content. By adjusting the UVB dose it would be possible to tailor the vitamin D content in liquid egg products. The penetration depth of UVB at 296 nm is 0.01-0.08 mm (Argyraki, 2017) therefore any practical application would have to be on a very thin film or in a dynamic treatment (e.g. stirring).

Dynamic treatment of liquid egg products with an UVC dose of 42000 J/m^2 obtained in 30 minutes has the same disinfectant properties as heat pasteurization but with only minor changes to the rheological properties (de Souza & Fernández, 2013). It has been shown that UVB is more effective than UVC in inactivating the bacteria *Pseudomonas aeruginosa* in a trial where the exact same UV-LED as used for UVB exposure of hens in (Argyraki, 2017) was used; a dose of 10,000 J/m^2 left no viable colonies, and the exposure time was less than 12 minutes (Argyraki, Markvart, Bjørndal, Bjarnsholt, & Petersen, 2017). Thereby, there is potential that a dynamic treatment with UVB, alone or in combination with UVC could produce both safe and vitamin D enriched liquid egg products (yolk and whole egg mix).

12. Concluding remarks and future trends

In Denmark the average intake of eggs per person per day is 25 g (Pedersen et al., 2015), in Ireland it is 16 g (O'Mahony et al., 2011). 10 % of males in Denmark ingest more than 55 g per day (Pedersen et al., 2015).

How effective 25(OH)D_3 is compared to vitamin D_3 in increasing serum 25(OH)D_3 depends on the dose of 25(OH)D_3. The factor between the efficiencies is 1.04 at 5 µg/day, 1.5-5.5 at doses below 25 µg/day and 8 at doses above 50 µg/day (reviewed by Quesada-Gomez & Bouillon (2018), (Jakobsen et al., 2018)). The daily intake of 25(OH)D_3 from food is assumed to be less than 5 µg/day, for this reason we have used factor 1 when calculating the total vitamin D content. Cooking loss and bioavailability have not been included in the calculation of the average intake of vitamin D from eggs.

According to the findings in Section 7 the average total vitamin D content in commercial eggs is 2 µg/100 g (using factor 1 for 25(OH)D_3, see above). The maximum obtainable total vitamin D content in eggs from hens exposed to UVB, directed at the legs (in cages), is 5.1 µg/100 g (see Section 9.3). If eggs in Denmark and Ireland were increased to have a vitamin D content of 5.1 µg/100 g the daily average intake per person would increase with 0.8 µg and 0.5 µg, respectively (the actual intake will be lower as cooking loss was not included in the calculations). However, implementing UVB-lamps directed at the feet of the hens in a barn will be a challenging task. If placed near the floor the lamps will get dirty and a vast amount of cleaning will be required. In addition only the hens nearby the lamp will be exposed and the dose each hen receives will be hard to control. If UVB-treatment is to be implemented in barn egg production facilities it will be necessary to develop solutions where the hens are irradiated from above to ensure even exposure and minimisation of costs for both maintenance and cleaning.

The gap between the recommended and the actual vitamin D intake is 3-17 µg/day/person and it therefore might be desirable to increase the vitamin D content of eggs further than what is possible with

UVB exposure. There is an inter-trial linear relationship between the vitamin D_3 content in the feed and the content of vitamin D_3 in the egg (see section 8.1) and within the linear range the highest content is 20 µg/100 g which is achieved with a vitamin D_3 content of 617.5 µg/kg feed. This level is well within the reported safe level for laying hens (Mattila et al., 2003, 2004; Persia et al., 2013). Feeding vitamin D_3 will also increase the content of 25(OH)D_3 in the egg, however the relationship between vitamin D_3 in feed and the 25(OH)D_3 is most likely logarithmic with a maximum around 1 µg/100 g (see Figure 2). The total vitamin D content in eggs from hens supplemented with 617.5 µg/kg feed would be 21 µg/100 g; such a level would increase of the daily average intake from eggs per person with 4.8 µg in Denmark and 3.0 µg in Ireland. Although this content might be too high, for a single source of vitamin D, it illustrates that it is possible to design the vitamin D_3 content to any desired level through feed.

The use of 25(OH)D_3 in feed was legalised in 2009 in Europe, and trials have shown that 25(OH)D_3 content of eggs will increase when 25(OH)D_3 is added to the feed; however, there is not the same linear inter-trial tendency as observed for vitamin D_3, and the dose-response is a factor 3 less compared to vitamin D_3. Also, 25(OH)D_3 is more toxic to hens than vitamin D_3 as 413 µg/kg feed is on the margin of being toxic (Terry et al., 1999) whereas no negative effects of vitamin D_3 has been observed with a dose of 2555 µg/kg feed (Yao et al., 2013).

Feeding a combination of 250 µg/kg feed of vitamin D_3 an 69 µg/kg feed of 25(OH)D_3 gives a total vitamin D content of 12.9 µg/100 g (Browning & Cowieson, 2014). To obtain the same level using vitamin D_3 alone would require approximately 378 µg/kg feed.

In order to increase the content of vitamin D in eggs through feed the European legislation has to be changed, as the current limit is 80 µg vitamin D/kg feed. Fortunately these limits are not set in stone; according to the procedure described in Regulation (EC) No 1831/2003 one can send an application with new information to the European Commission (using the application form found in Commission Regulation (EC) No 492/2008) the Commission shall send it to EFSA who shall provide a new opinion. If the opinion is positive it will most likely result in a draft Regulation made by the Standing Committee on Plant, Animals, Food and Feed (PAFF committee - section animal nutrition), on behalf of the European Commission, who will also decide if the draft Regulation should eventually be put into force. The Norwegian Food Safety Authority (NFSA) applied for increasing the maximum limit 20 times, from 75 µg/kg feed to 1.5 mg/kg feed for salmonids and received a positive opinion from EFSA (EFSA, 2017, 2019). The opinion is currently being discussed at PAFF committee meetings (Standing Committee on Plant, Animals, 2019).

If choosing between feed and UVB exposure for natural enhancement of eggs with vitamin D, increasing the vitamin D_3 in the feed seems to give the most predictable and cost effective results as only the feed has to be changed. A third option is to expose liquid egg products directly to UVB. With this method the vitamin D content can be tailored by adjusting the dose, and in addition the bacterial load is reduced due

to the dual effect of UVB; however further research and development of equipment is needed for this to be implemented and approved as a novel food.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Ameenuddin, S. (1986). Excessive cholecalciferol in a layers diet: decline in some aspects of reproductive performance and increased bone mineralisation of progeny. *British Poultry Science*, *27*(4), 671–677.

Argyraki, A. (2017). *New light Sources for Biomedical Applications*. Technical University of Denmark (DTU). Retrieved from http://orbit.dtu.dk/en/publications/new-light-sources-for-biomedical-applications(4c1e9ea9-6508-4134-ba60-00fd50d1f00d).html

Argyraki, A., Markvart, M., Bjørndal, L., Bjarnsholt, T., & Petersen, P. M. (2017). Inactivation of Pseudomonas aeruginosa biofilm after ultraviolet light-emitting diode treatment: a comparative study between ultraviolet C and ultraviolet B. *Journal of Biomedical Optics*, *22*(6), 065004. http://doi.org/10.1117/1.jbo.22.6.065004

Barnkob, L. L., Argyraki, A., Petersen, P. M. P. M., & Jakobsen, J. (2016). Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin. *Food Chemistry*, *212*, 386–391. http://doi.org/10.1016/j.foodchem.2016.05.155

BASF. (1998). *Vitamin Supplementation Rates for U.S. Commercial Poultry, Swine and Dairy Cattle, 1997. KC 9305. 2nd Revised Edition*. Retrieved from http://www.advit.co.za/downloads/BASF KC 9305.pdf

Bethke, R. M., Kennard, D. C., & Sassaman, H. L. (1927). The fat-soluble vitamin content of hen's egg yolk as affected by the ration and management of the layers. *Journal of Biological Chemistry*, *72*, 695–706.

Black, L. J., Seamans, K. M., Cashman, K. D., & Kiely, M. (2012). An Updated Systematic Review and Meta-Analysis of the Efficacy of Vitamin D Food Fortification. *Journal of Nutrition*, *142*(6), 1102–1108. http://doi.org/10.3945/jn.112.158014

Borel, P., Caillaud, D., & Cano, N. J. (2015). Vitamin D Bioavailability: State of the Art. *Critical Reviews in Food Science and Nutrition*, *55*(9), 1193–1205. http://doi.org/10.1080/10408398.2012.688897

Branion, H. D., Drake, T. G., & Tisdall, F. F. (1935). The vitamin D content of egg yolk. *Canadian Medical*

Association Journal, *32*(1), 9–12.

Browning, L. C., & Cowieson, A. J. (2014). Vitamin D fortification of eggs for human health. *Journal of the Science of Food and Agriculture*, *94*(7), 1389–96. http://doi.org/10.1002/jsfa.6425

Carson, J. R., & Beall, G. (1955). Absence of Response by Breeder Hens to Ultraviolet Energy. *Poultry Science*, *34*(2), 256–262.

Cashman, K. D. (2015). Vitamin D: dietary requirements and food fortification as a means of helping achieve adequate vitamin D status. *Journal of Steroid Biochemistry and Molecular Biology*, *148*, 19–26. http://doi.org/10.1016/j.jsbmb.2015.01.023

Cashman, K. D., Dowling, K. G., Gonzalez-Gross, M., Valtue, J., Henauw, S. De, Moreno, L., … Kiely, M. (2016). Vitamin D deficiency in Europe: pandemic? *American Journal of Clinical Nutrition*, *103*, 1033–1044. http://doi.org/10.3945/ajcn.115.120873

Cashman, K. D., & Kiely, M. (2016). Tackling inadequate vitamin D intakes within the population: fortification of dairy products with vitamin D may not be enough. *Endocrine*, *51*(1), 38–46. http://doi.org/10.1007/s12020-015-0711-x

Chiang, Y. H., Hwang, S. I., & Holick, M. F. (1996). Influence of different time of ultraviolet irradiation on performance and vitamin D-3 metabolism in laying hens. *Korean Journal of Animal Nutrition and Feedstuffs*, *20*(6), 497–508.

Chiang, Y. H., Hwang, S. I., & Holick, M. F. (1997). Effect of vitamin D_3 oral dose or ultraviolet irradiation on yolk vitamin D3, plasma minerals, eggshell thickness and intensity in laying hens. *Korean Journal of Animal Nutrition and Feedstuffs*, *21*(6), 475–488.

Christakos, S., Dhawan, P., Verstuyf, A., Verlinden, L., & Carmeliet, G. (2016). Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev*, *96*, 365–408. http://doi.org/10.1152/physrev.00014.2015.-1,25-Dihydrox

de Souza, P. M., & Fernández, A. (2013). Rheological properties and protein quality of UV-C processed liquid egg products. *Food Hydrocolloids*, *31*(1), 127–134. http://doi.org/10.1016/j.foodhyd.2012.05.013

DeVaney, G. M., Munsell, H. E., & Titus, H. W. (1933). Effect of Sources of Vitamin D on Storage of the Antirachitic Factor in the Egg. *Poultry Science*, *12*(4), 215–222. http://doi.org/10.3382/ps.0120215

DTU Food. (2018). Frida Food Data, rel. 3. Retrieved January 10, 2019, from http://frida.fooddata.dk

DTU Library. (n.d.). DTU Findit (DTU version): About DTU Findit. Retrieved April 3, 2019, from http://libguides.dtu.dk/c.php?g=654792&p=4600996

Duffy, S. K., Rajauria, G., Clarke, L. C., Kelly, A. K., Cashman, K. D., & O'Doherty, J. V. (2017). The potential of cholecalciferol and 25-hydroxyvitamin D3 enriched diets in laying hens, to improve egg vitamin D content and antioxidant availability. *Innovative Food Science & Emerging Technologies*. http://doi.org/10.1016/j.ifset.2017.07.007

Edwards, H. M. (2003). Effects of u.v. irradiation of very young chickens on growth and bone development. *British Journal of Nutrition*, *90*, 151. http://doi.org/10.1079/BJN2003860

EFSA. (2005). Opinion of the Scientific Panel on additives and products or substances used in animal feed (FEEDAP) on the evaluation of safety and efficacy of "Hy•D" (calcifediol), based on 25-hydroxylcholecalciferol/25-hydroxy-pre-cholecalciferol, as feed additive in. *EFSA Journal*, *3*(7), 224. http://doi.org/10.2903/j.efsa.2005.224

EFSA. (2014). Scientific Opinion on the safety and efficacy of vitamin D3 (cholecalciferol) as a feed additive for all animal species or categories based on a dossier submitted by Lohmann Animal Health GmbH. *EFSA Journal*, *12*(2), 3568. http://doi.org/10.2903/j.efsa.2014.3568

EFSA. (2017). Safety of vitamin D_3 addition to feedingstuffs for fish. *EFSA Journal*, *15*(3). http://doi.org/10.2903/j.efsa.2017.4713

EFSA. (2019). Safety for the environment of vitamin D_3 for salmonids. *EFSA Journal*, *17*(1). http://doi.org/10.2903/j.efsa.2019.5540

Farrell, F. (1924). Relation of vitamin content of feed and of the use of light to immunity to roup and the production and hatchability of eggs. *Director's Report 1922-1924 for the Biennium. Agricultiural Experiment Station, Kansas State Agricultural College, Manhatten, Kansas*, 113–116.

Francis G. McDonald, & Massengale, O. N. (1932). The antiricketic potenct of eggs from hens receiving massive doses of activated ergosterol. *J. Biol. Chem.*, *99*(1), 79–83.

Fraser, D. R., & Emtage, J. S. (1976). Vitamin D in the Avian Egg - its molecular identity and mechanism of incorporation into yolk. *Biochem J*, *160*, 671–682.

Gray, J. (2018). British eggs: Back on the menu for all. *Nutrition Bulletin*, *43*(1), 85–92. http://doi.org/10.1111/nbu.12310

Guerrant, N. B., Kohler, E., Hunter, J. E., & Murphy, R. R. (1935). The Relationship of the Vitamin D Intake

of the Hen to the Antirachitic Potency of the Eggs Produced. *The Journal of Nutrition*, *10*(2), 167–178.

Hart, E., Steenbock, H., Lepkovsky, S., Kletzien, S. W. F., Halpin, J. G., & Johnson, O. N. (1925). The nutritional requirement of the chicken. V. The influence of ultra-violet light on the production, hatchability, and fertility of the egg. *Journal of Biological Chemistry*, *65*(3), 579–595.

Hayes, A., Duffy, S., OGrady, M., Jakobsen, J., Galvin, K., Teahan-Dillon, J., … Cashman, K. D. (2016). Vitamin D-enhanced eggs are protective of wintertime serum 25-hydroxyvitamin D in a randomized controlled trial of adults. *American Journal of Clinical Nutrition*, *104*(3), 629–637. http://doi.org/10.3945/ajcn.116.132530

Hedegaard. (n.d.). Livskraft. Retrieved April 3, 2019, from http://www.hedegaard-foods.dk/livskraft

Hendricks, W. (1931). Effects of cod-liver oil and ultra-violet irradiation, as influenced by oyster shell, in the diet of confined laying hens. *Journal of Agricultural Research*, *43*, 0517–0535.

Holick, M. F. (2017). The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. *Rev Endocr Metab Disord*, *18*(2), 153–165. http://doi.org/10.1007/s11154-017-9424-1

Holick, M. F., MacLaughlin, J. A., Clark, M. B., Holic, S. A., Potts, T. J., Anderson, R. R., … Elias, P. (1980). Photosynthesis of Previtamin D_3 in Human Skin And the Physiologic Consequences. *Science*, *210*(4466), 203–205. http://doi.org/10.1126/science.6251551

Holvik, K., Madar, A. A., Meyer, H. E., Lofthus, C. M., & Stene, L. C. (2007). A randomised comparison of increase in serum 25-hydroxyvitamin D concentration after 4 weeks of daily oral intake of 10 µg cholecalciferol from multivitamin tablets or fish oil capsules in healthy young adults. *British Journal of Nutrition*, *98*(3), 620–625. http://doi.org/10.1017/S000711450773074X

Hughes, J., & Payne, L. (1924). Influence of ultra-violet light on young laying hens. *Science*, *60*(1563), 549–50.

Hughes, J., Payne, L., Titus, R., & Moore, J. (1925). The relation between the amount of ultraviolet light received by hens and the amount of antirachitic vitamin in the eggs produced. *Journal of Biological Chemistry*, *66*(2), 595–600.

Institute of Medicine. (2011). *Dietary Reference Intakes for Vitamin D and Calcium*. Washington DC: The National Academies Press. http://doi.org/10.17226/13050

Jääskeläinen, T., Itkonen, S. T., Lundqvist, A., Erkkola, M., Koskela, T., Lakkala, K., … Lamberg-Allardt, C. (2017). The positive impact of general Vitamin D food fortification policy on Vitamin D status in a

representative adult Finnish population: Evidence from an 11-y follow-up based on standardized 25-hydroxyVitamin D data. *American Journal of Clinical Nutrition*, *105*(6), 1512–1520. http://doi.org/10.3945/ajcn.116.151415

Jakobsen, J., Andersen, E., Christensen, T., Andersen, R., Bügel, S., Jakobsen, J., … Bügel, S. (2018). Vitamin D Vitamers Affect Vitamin D Status Differently in Young Healthy Males. *Nutrients*, *10*(1), 12. http://doi.org/10.3390/nu10010012

Jakobsen, J., & Knuthsen, P. (2014). Stability of vitamin D in foodstuffs during cooking. *Food Chemistry*, *148*, 170–175. http://doi.org/10.1016/j.foodchem.2013.10.043

Kawazoe, T., Yuasa, K., Noguchi, K., Yamazaki, M., & Ando, M. (1996). Effect of different sources of vitamin D on transfer of vitamin D to egg yolk. *Nippon Shokuhin Kagaku Kogaku Kaishi*, *43*(4), 444–450. http://doi.org/https://doi.org/10.3136/nskkk.43.444

Kawazoe, T., Yuasa, K., Yamazaki, M., & Ando, M. (1994). Production of Vitamin D-fortified eggs by feeding vitamin D2-fortified shiitake. *Nippon Shokuhin Kagaku Kogaku Kaishi*, *41*(12), 891–896. http://doi.org/https://doi.org/10.3136/nskkk1962.41.891

Keegan, R. J. H., Lu, Z., Bogusz, J. M., Williams, J. E., & Holick, M. F. (2013). Photobiology of vitamin D in mushrooms and its bioavailability in humans. *Dermato-Endocrinology*, *5*(1), 165–176. http://doi.org/10.4161/derm.23321

Kiely, M., & Black, L. J. (2012). Dietary strategies to maintain adequacy of circulating 25-Hydroxyvitamin D concentrations. *Scandinavian Journal of Clinical and Laboratory*, *72*, 14–23. http://doi.org/10.3109/00365513.2012.681893

Koch, E. M., & Koch, F. C. (1941). The Provitamin D of the Covering Tissues of Chickens. *Poultry Science*, *20*(1), 33–35. http://doi.org/10.3382/ps.0200033

Koshy, K. T., & Van Der Slik, A. L. (1979). High-performance liquid chromatographic method for the determination of 25-hydroxycholecalciferol in chicken egg yolks. *Journal of Agricultural and Food Chemistry*, *27*(1), 180–183. http://doi.org/10.1021/jf60221a033

Kühn, J., Schutkowski, A., Hirche, F., Baur, A. C., Mielenz, N., & Stangl, G. I. (2015). Non-linear increase of vitamin D content in eggs from chicks treated with increasing exposure times of ultraviolet light. *The Journal of Steroid Biochemistry and Molecular Biology*, *148*, 7–13. http://doi.org/10.1016/j.jsbmb.2014.10.015

Kühn, J., Schutkowski, A., Kluge, H., Hirche, F., & Stangl, G. I. (2014). Free-range farming: A natural

alternative to produce vitamin D-enriched eggs. *Nutrition*, *30*(4), 481–484. http://doi.org/10.1016/j.nut.2013.10.002

Lehtonen-Veromaa, M., Möttönen, T., Leino, A., Heinonen, O. J., Rautava, E., & Viikari, J. (2008). Prospective study on food fortification with vitamin D among adolescent females in Finland: minor effects. *The British Journal of Nutrition*, *100*(2), 418–23. http://doi.org/10.1017/S0007114508894469

Lietzow, J., Kluge, H., Brandsch, C., Seeburg, N., Hirche, F., Glomb, M., & Stangl, G. I. (2012). Effect of short-term UVB exposure on vitamin D concentration of eggs and vitamin D status of laying hens. *Journal of Agricultural and Food Chemistry*, *60*(3), 799–804. http://doi.org/10.1021/jf204273n

Liu, J., Greenfield, H., & Fraser, D. R. (2014). An exploratory study of the content of vitamin D compounds in selected samples of Australian eggs. *Nutrition & Dietetics*, *71*(1), 46–50. http://doi.org/10.1111/1747-0080.12056

MacLaughlin, J. A., Anderson, R. R., & Holick, M. F. (1982). Spectral character of sunlight modulates photosynthesis of previtamin D_3 and its photoisomers in human skin. *Science*, *216*(4549), 1001–1003. http://doi.org/10.1126/science.6281884

Mattila, P., Lehikoinen, K., Kiiskinen, T., & Piironen, V. (1999). Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. *Journal of Agricultural and Food Chemistry*, *47*(10), 4089–4092. http://doi.org/10.1021/jf990183c

Mattila, P., Rokka, T., Könkö, K., Valaja, J., Rossow, L., & Ryhänen, E.-L. (2003). Effect of cholecalciferol-enriched hen feed on egg quality. *Journal of Agricultural and Food Chemistry*, *51*(1), 283–7. http://doi.org/10.1021/jf020743z

Mattila, P., Ronkainen, R., Lehikoinen, K., & Piironen, V. (1999). Effect of household cooking on the vitamin D content in fish, eggs, and wild mushrooms. *Journal of Food Composition and Analysis*, *12*, 153–160. http://doi.org/https://doi.org/10.1006/jfca.1999.0828

Mattila, P., Valaja, J., Rossow, L., Venäläinen, E., & Tupasela, T. (2004). Effect of vitamin D2- and D3-enriched diets on egg vitamin D content, production, and bird condition during an entire production period. *Poultry Science*, *83*(3), 433–40. http://doi.org/10.1093/ps/83.3.433

Mattila, P., Valkonen, E., & Valaja, J. (2011). Effect of different vitamin D supplementations in poultry feed on vitamin D content of eggs and chicken meat. *Journal of Agricultural and Food Chemistry*, *59*(15), 8298–303. http://doi.org/10.1021/jf2012634

Maughan, G. H., & Maughan, E. (1933). The vitamin D potency of egg yolk from irradiated hens. *Science*,

77(1990), 198. http://doi.org/10.1126/science.77.1990.198

Mejborn, H., Jacobsen, S. M., & Trolle, E. (2011). Æg i kosten og betydningen for sundhed og sygdom. Søborg. Retrieved from http://www.food.dtu.dk/english/publications/nutrition

Nordic Council of Ministers. (2012). Nordic Nutrition Recommendations 2012 : Integrating nutrition and physical activity. Nordisk Ministerråd.

O'Mahony, L., Stepien, M., Gibney, M. J., Nugent, A. P., & Brennan, L. (2011). The potential role of vitamin D enhanced foods in improving vitamin D status. *Nutrients*, *3*(12), 1023–41. http://doi.org/doi:10.3390/nu3121023

O'Neill, C., Kazantzidis, A., Ryan, M., Barber, N., Sempos, C., Durazo-Arvizu, R., … Cashman, K. D. (2016). Seasonal Changes in Vitamin D-Effective UVB Availability in Europe and Associations with Population Serum 25-Hydroxyvitamin D. *Nutrients*, *8*(9), 533. http://doi.org/10.3390/nu8090533

Ovesen, L., Brot, C., & Jakobsen, J. (2003). Food contents and biological activity of 25-hydroxyvitamin D: A vitamin D metabolite to be reckoned with? *Annals of Nutrition and Metabolism*, *47*(3–4), 107–113.

Park, S. W., Namkung, H., Ahn, H. J., & Paik, I. K. (2005). Enrichment of Vitamins D_3, K and Iron in Eggs of Laying Hens. *Asian-Australasian Journal of Animal Sciences*, *18*(2), 226–229.

Pedersen, A., Christensen, T., Matthiesen, J., Knudsen, V. B., Rosenlund-Sørensen, M., Biltoft-Jensen, A., … Fagt, S. (2015). *Dietary habits in Denmark 2011-2013. Main results*. Søborg. Retrieved from http://www.food.dtu.dk/english/Publications/Nutrition/Danish-National-Survey-of-Dietary-Habits-and-Physical-Activity

Persia, M. E., Higgins, M., Wang, T., Trample, D., & Bobeck, E. a. (2013). Effects of long-term supplementation of laying hens with high concentrations of cholecalciferol on performance and egg quality. *Poultry Science*, *92*(11), 2930–7. http://doi.org/10.3382/ps.2013-03243

Plaimast, H., Kijparkorn, S., & Ittitanawong, P. (2015). Effects of vitamin D_3 and calcium on productive performance, egg quality and vitamin D_3 content in egg of second production cycle hens. *Thai Journal of Veterinary Medicine*, *45*(2), 189–195. http://doi.org/10.1590/S1516-635X2011000300005

Quesada-Gomez, J. M., & Bouillon, R. (2018). Is calcifediol better than cholecalciferol for vitamin D supplementation? *Osteoporosis International*, *29*(8), 1697–1711. http://doi.org/10.1007/s00198-018-4520-y

Reboul, E. (2015). Intestinal absorption of vitamin D: from the meal to the enterocyte. *Food & Function*, *6*,

356–362. http://doi.org/10.1039/c4fo00579a

Schutkowski, A., Krämer, J., Kluge, H., Hirche, F., Krombholz, A., Theumer, T., & Stangl, G. I. (2013). UVB exposure of farm animals: study on a food-based strategy to bridge the gap between current vitamin D intakes and dietary targets. *PloS One*, *8*(7), e69418.

Standing Committee on Plant, Animals, F. and F. S. A. N. (2019). *Standing Committee on Plants, Animals, Food and Feed Section Animal Nutrition. 09-11 April 2019. Agenda*. Retrieved from https://ec.europa.eu/food/animals/committees/sc_animal-nutrition_en

Świątkiewicz, S., Arczewska-Wlosek, A., Bederska-Lojewska, D., & Józefiak, D. (2017). Efficacy of dietary vitamin D and its metabolites in poultry - review and implications of the recent studies. *World's Poultry Science Journal*, *73*(01), 57–68. http://doi.org/10.1017/S0043933916001057

Terry, M., Lanenga, M., McNaughton, J. L., & Stark, L. E. (1999). Safety of 25-hydroxyvitamin D_3 as a source of vitamin D_3 in layer poultry feed. *Veterinary and Human Toxicology*, *41*(5), 312–6.

Tian, X. Q., Chen, T. C., Lu, Z., Shau, Q., & Holick, M. F. (1994). Characterization of the translocation process of vitamin D3 from the skin into the circulation. *Endocrinology*, *135*(2), 655–661.

Torbergsen, A. C., Watne, L. O., Wyller, T. B., Frihagen, F., Strømsøe, K., Bøhmer, T., & Mowe, M. (2015). Vitamin K1 and 25(OH)D are independently and synergistically associated with a risk for hip fracture in an elderly population: A case control study. *Clinical Nutrition*, *34*(1), 101–106. http://doi.org/10.1016/j.clnu.2014.01.016

Uva, B., Mandich, A., & Vallarino, M. (1983). The site of 7-dehydrocholesterol ultraviolet photolysis in domestic fowls. *Acta Histochemica*, *73*(2), 175–180.

Webb, A. R. (2006). Who, what, where and when-influences on cutaneous vitamin D synthesis. *Progress in Biophysics and Molecular Biology*, *92*(1), 17–25. http://doi.org/10.1016/j.pbiomolbio.2006.02.004

Webb, A. R., DeCosta, B. R., & Holick, M. F. (1989). Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation. *Journal of Clinical Endocrinology and Metabolism*, *68*(5), 882–887. http://doi.org/10.1210/jcem-68-5-882

Webb, A. R., Kline, L., & Holick, M. F. (1988). Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D_3 synthesis in human skin. *The Journal of Clinical Endocrinology and Metabolism*, *67*(2), 373–8.

Whiting, S. J., Green, T. J., & Calvo, M. S. (2007). Vitamin D intakes in North America and Asia-Pacific

countries are not sufficient to prevent vitamin D insufficiency. *The Journal of Steroid Biochemistry and Molecular Biology*, *103*(3–5), 626–30. http://doi.org/10.1016/j.jsbmb.2006.12.067

Yao, L., Wang, T., Persia, M., Horst, R. L., & Higgins, M. (2013). Effects of vitamin D_3-enriched diet on egg yolk vitamin D3 content and yolk quality. *Journal of Food Science*, *78*(2), C178-83. http://doi.org/10.1111/1750-3841.12032

Zang, H., Zhang, K., Ding, X., Bai, S., Hernández, J. M., & Yao, B. (2011). Effects of Different Dietary Vitamin Combinations on the Egg Quality and Vitamin deposition in the Whole Egg of Laying Hens. *Brazilian Journal of Poultry Science*, *13*(3), 189–196. http://doi.org/10.1590/S1516-635X2011000300005

Paper IV

1 **Deuterated internal standard in the analysis of vitamin D in foods does not always correct for matrix**
2 **effects: the effect of eluent additives**

3 Line Lundbæk Barnkob[a], Henrik Lauritz Frandsen[b] and Jette Jakobsen[a]*

4

5 [a] National Food Institute, Technical University of Denmark, Research Group for Bioactives – Analysis and

6 Application, Kemitorvet, 2800 Kgs. Lyngby, Denmark

7 [b] National Food Institute, Technical University of Denmark, Research Group for Analytical Food Chemistry,

8 Kemitorvet, 2800 Kgs. Lyngby, Denmark

9 * Corresponding author

10 E-mail: jeja@food.dtu.dk (J. Jakobsen)

11 **Abstract**

12 It is generally assumed that using a stable isotope labelled internal standard (SIL IS) will remove any relative
13 matrix effect (ME). We use deuterated vitamin D_3 as the internal standard when we analyse vitamin D_3 in
14 foods by LC-ESI-MS/MS. When we changed the eluent additive from methylamine/formic acid to
15 ammonium formate the accuracy and robustness of the method for some matrices was changed beyond
16 normal variation between methods. Using ammonium formate/formic acid as additive instead improved
17 the accuracy but not the robustness. Deuterated SIL IS are a little less lipophilic than the corresponding
18 analyte and therefore elutes slightly before the analyte. This difference in retention time (RT) can result in a
19 different degree of ion suppression of the two analogues due to ME affecting the area ratio and thereby
20 the determined content. Post-column infusion experiments can be used to visualise ME but requires a
21 matrix blank; however, this is hard to come by when analysing nutrients in food. So we came up with the
22 idea of using continuous post-column infusion of vitamin D_3-d_6 to visualise ion suppression when no blank
23 matrix is available. We visualised the effect different eluent additives have on ion suppression in extracts of
24 different food matrices. With a sample preparation consisting of saponification, liquid-liquid extraction,
25 SPE and derivatisation with PTAD we observed an enormous amount of ion suppression. Due to its earlier
26 RT deuterated SIL IS could not fully eliminate ME when analysing vitamin D_3 in most food matrices. To get
27 the accuracy to be completely independent of ME would require a more extensive sample clean-up or
28 introduce a ^{13}C SIL IS in the method.

29

30 **Keywords**

31 electrospray ionization mass spectrometry, post-column infusion, ion suppression, vitamin D, additives

32

33

34 **Highlights**

35 - Continuous post-column infusion of vitamin D_3-d_6 can be used to visualise ion suppression when no
36 blank matrix is available
37 - Use of vitamin D_3-d_6 does not guarantee correction of matrix effects in food matrices
38 - Eluent additives affect the ionisation of vitamin D_3

39

1. Introduction

In our accredited laboratory we have been analysing vitamin D vitamers by chemical methods in food samples for more than 20 years. Originally the main purpose was to establish new data for the Danish Fooddata Bank, however in later years our expertise has also been used in several research projects. Through the years there has been a continuous development of the chemical methods. The first chemical methods were based on HPLC-UV and used large sample amounts, extensive sample clean-up with liquid-liquid extraction (LLE) using vast amounts of solvents and preparative HPLC. Vitamin D_2 is used as internal standard for vitamin D_3 and vice versa when using UV detectors. HPLC-UV methods are precise and accurate when a diode array detector (DAD) is used to provide a spectrum that show a pure peak; extensive sample clean-up is necessary in order to achieve a pure spectrum of vitamin D. HPLC-UV methods are however labour intensive, time consuming, up to 200 g of sample is necessary to achieve a satisfactory limit of quantification (LOQ) and only a limited number of samples can be analysed per week. As LC instruments coupled to a mass spectrometer (MS) became more common in research laboratories methods using LC-MS with atmospheric pressure chemical ionisation (APCI) for the analysis of vitamin D emerged. APCI have been used both with MS and tandem MS (MS/MS). For the purpose of enhancing the sensitivity and thereby decreasing the LOQ derivatisation methods emerged using mainly 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). PTAD increases the polarity of the molecule and thereby its ionisation efficiency making it possible to use electrospray ionisation (ESI) instead of APCI.

LC-MS methods for the analysis of vitamin D in food mostly consist of a saponification step, a liquid-liquid extraction (LLE) step and a solid-phase-extraction (SPE) step. LC-ESI-MS methods typically also include derivatisation with PTAD, use vitamin D_3-d_6 as internal standard and the eluent additives are formic acid alone or in combination with methylamine or ammonium formate alone (Barnkob, Petersen, Nielsen, & Jakobsen, 2019; Burild, Frandsen, Poulsen, & Jakobsen, 2014; Gomes, Shaw, Whitfield, & Hewavitharana, 2015; Lietzow et al., 2012; Lipkie et al., 2013; Schadt, Gössl, Seibel, & Aebischer, 2012) while the LC-APCI-

65 MS methods either uses vitamin D_3-d_3 or vitamin D_2 as internal standard (Bilodeau et al., 2011; W Craig
66 Byrdwell et al., 2008; William C. Byrdwell et al., 2013; Dimartino, 2009; Gilliland, Black, Denison, Seipelt, &
67 Dowell, 2012; Heudi, Trisconi, & Blake, 2004; Höller et al., 2010; Huang, Winters, Sullivan, & Dowell, 2012;
68 Stevens & Dowell, 2012; Strobel, Buddhadasa, Adorno, Stockham, & Greenfield, 2013; Trenerry, Plozza,
69 Caridi, & Murphy, 2011).

70 Compounds with the same retention time, precursor and product ion are seldom the cause of
71 interferences in LC-MS/MS. Interferences are rather caused by other co-eluting compounds that influence
72 the ionisation of the analyte (Bonfiglio et al., 1999). It is known that co-eluting matrix compounds may
73 cause ion suppression or ion enhancement although the underlying mechanism of the matrix effect (ME) is
74 not yet fully elucidated (Matuszewski, Constanzer, & Chavez-Eng, 2003). It is generally assumed that a
75 stable isotope labelled internal standard (SIL IS) will remove any relative ME as it is presumed that it co-
76 elute and behave physically and chemically like the analyte (Bonfiglio et al., 1999; Matuszewski, 2006;
77 Matuszewski et al., 2003; Wu, Wiegand, Lorusso, & Li, 2013). However, deuterated (^{2}H) SIL ISs are a little
78 less lipophilic than the corresponding analyte and therefore elutes slightly before the analyte. This
79 difference in retention time (RT) can result in a different degree of ion suppression of the two analogues
80 due to ME affecting the area ratio and thereby the determined content (Wang, Cyronak, & Yang, 2007;
81 Wieling, 2006). When using MRM (multiple reaction monitoring) or SRM (selected reaction monitoring) in
82 MS/MS only the peaks of interest are observed, however interferences not detected can still have an
83 impact on the signal. In the case of suppression the LOD will be decreased (Buhrman, Price, & Rudewicz,
84 1996) and if IS and analyte does not co-elute it can affect the accuracy (correctness in ISO) of the method.

85 Reviews of methods for evaluating ME have been published elsewhere (Furey, Moriarty, Bane,
86 Kinsella, & Lehane, 2013; Trufelli, Palma, Famiglini, & Cappiello, 2011). Using recovery based methods for
87 detecting interferences does not give information on the chromatographic profile of the interference or of
88 late eluting interferences that if not eluted can affect subsequent runs (Bonfiglio et al., 1999). A full scan of
89 a sample can sometimes give information about interfering compounds, however interferences cannot

90 necessarily be detected by MS (Bonfiglio et al., 1999). The only method that also includes the
91 chromatographic profile is the post-column infusion method which was first described by (Bonfiglio et al.,
92 1999). The post-column infusion method requires a matrix blank; however, this is hard to come by when
93 analysing nutrients in food. We assumed that disregarding the chromatographic separation, ME will affect
94 the ionisation of deuterated vitamin D_3 (vitamin D_3-d_6) and vitamin D_3 in the same manner; so we came up
95 with the idea of using continuous post-column infusion of vitamin D_3-d_6 to visualise ion suppression when
96 no blank matrix is available.

97 When we changed our eluent additive from methylamine/formic acid to ammonium formate the
98 accuracy and robustness our method was not satisfactory; the content of vitamin D_3 was overestimated by
99 approximately 30 % in egg yolk even though we used vitamin D_3-d_6 as IS. Using a combination of
100 ammonium formate/formic acid increased the accuracy but not the robustness. The aim of this study was
101 therefore to visualise how ME is affected by eluent additives in order to find an explanation behind our
102 observations.

103

104 **2. Experimental**

105

106 *2.1. Chemicals and reagents*

107

108 The following chemicals were purchased from Sigma-Aldrich (Steinheim, Germany): Anhydrous
109 acetonitrile, 99.8 %; Fluka Formic acid for MS; 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD); Ammonium
110 formate. Vitamin D_3-[26,26,26,27,27,27-d_6], was purchased from Chemaphor Inc. (Ottawa, Canada). HPLC-
111 grade ethyl acetate and 2-propanol were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland).
112 N-heptane (LC grade), methanol (Honeywell, LC-MS grade), ethanol (96%), sodium ascorbate and
113 potassium hydroxide were purchased from Merck (Darmstadt, Germany). Milli-Q water was made in-house
114 (18.2 MΩ, Millipore, Billerica, MA).

115

116 *2.2. Samples*

117

118 One certified reference material and 6 in-house reference samples were chosen for the study: 1546a

119 (SRM®1546a, meat homogenate (www.nist.gov/srm); however, the vitamin D content is not certified but is

120 determined to be 2.33±0.034 ng/g in an inter-laboratory comparison (Roseland et al., 2016)), pork loin

121 (from 2006), beef (2016), pork loin (2016), pork fat (2014), salmon (2014), pork liver (2014) and pasteurised

122 egg yolk (2012/2015; reference from 2012 were used up however the vitamin D_3 content in reference from

123 2015 was the same). All in-house reference samples are bought from local supermarket, homogenized,

124 divided into portions of approximately 10 g and stored at -20 °C.

125

126 *2.3 Analytical methods*

127

128 *2.3.1. HPLC-UV-DAD*

129 A full description of the HPLC-UV-DAD method is publish elsewhere (Jakobsen, Clausen, Leth, & Ovesen,

130 2004; Jakobsen, Maribo, Bysted, Sommer, & Hels, 2007; Jakobsen & Saxholt, 2009). In short, 10-200 g of

131 sample was saponified, extracted using LLE with petroleum ether:diethylether (300 mL in total) followed by

132 silica SPE (90 mL n-heptan/2-propanol in total) final clean-up step was semi-preparative HPLC before the

133 extract was injected into the HPLC-UV-DAD. Vitamin D was detected using DAD (220-320 nm) and

134 quantified using UV (265 nm). Vitamin D_2 was used as IS.

135

136 *2.3.2. LC-ESI-MS/MS – methylamine/formic acid*

137

138 The method is published elsewhere (Burild et al., 2014). In short, 1 gram of sample was saponified,

139 extracted by LLE using ethyl acetat:n-heptan (total 30 mL), followed by silica SPE (12 mL n-heptan/2-

140 propanol in total). Derivatised using PTAD before injection on HPLC-MS/MS. The eluent was A: Milli-Q-

141 water and B: Methanol (MeOH) with 5 mM methylamine and 0.1 % formic acid. The methylamine adduct

142 was used as precursor ion and the product ion with m/z 298 was used for quantification using MRM

143 (multiple reaction mode). Vitamin D_3-d_6 was used as IS.

144

145 *2.3.3. LC-ESI-MS/MS – ammonium formate*

146 A full description of the method is published elsewhere (Barnkob et al., 2019). In short, 1 gram of sample

147 was saponified, extracted by LLE using ethyl acetat:n-heptan (total 30 mL), followed by HybridSPE (1,5 mL

148 acetonitrile/formic acid in total). Derivatised using PTAD before injection on HPLC-MS/MS. The eluent was

149 A: Milli-Q-water with 5 % MeOH, B: MeOH, 2.5 mM ammonium formate. The protonated adduct was used

150 as precursor ion and the product ion with m/z 298 was used for quantification using MRM (multiple

151 reaction mode). Vitamin D_3-d_6 was used as IS.

152 The same method was used for determining the effect eluent additives have on adduct formation where

153 the eluent additives were changed.

154

155 *2.3.4. Post-column infusion*

156 2.3.4.1. Sample matrix preparation

157 The samples described in section 2.2. and a blank were treated according to the sample clean-up

158 described in (Barnkob et al., 2019) except that internal standard mixture was not added. In short, 1 gram of

159 sample was saponified overnight, cleaned-up by LLE subsequently by HybridSPE and finally derivatised

160 using PTAD. The extracts were stored at – 20 °C and kept at 5 °C in the auto sampler during runs.

161

162 2.3.4.2. Derivatised SIL-IS solution for continuous post-column infusion

163 50 µl of the 50 µg/mL solution vitamin D_3-[26,26,26,27,27,27-d_6] were evaporated to dryness under

164 nitrogen and re-dissolved in 2 mL of 0.3 mg/mL PTAD in anhydrous acetonitrile. It was mixed and left to

165 derivatise in darkness for 5 minutes and 0.6 mL of Milli-Q water were added. The solution was transferred
166 to a 50 mL volumetric flask and filled with methanol. The final concentration was 50 ng/mL.
167
168 2.3.4.3. HPLC–MS/MS conditions and continuous post-column infusion
169 Chromatographic separation of the extracts described in section 2.3 were performed on an Agilent
170 1200 Series HPLC; the column was an Ascentis Express C18 (2.1 mm X 10 cm, 2.7 µm particles) coupled with
171 an Ascentis Express C18 guard column (2.1 mm X 5 mm, 2.7 µm particles) from Supelco Analytical
172 (Bellafonte, PA) thermostated at 50 °C. Mobile phase A was water:methanol (95:5) with 2.5 mM
173 ammonium formate and 0 % or 0.05 % formic acid. Mobile phase B was methanol with 2.5 mM ammonium
174 formate and 0 % or 0.05 % formic acid. The flow was 0.4 mL/min and the gradient was as follows: 0-1.5
175 min, 75 %B; 1.5-1.6 min, linear gradient to 85 %B; 1.6-3.5 min, 85 %B; 3.5-3.6 min, linear gradient to 100
176 %B; 3.6-12 min, 100 %B; 12-12.1 min, linear gradient to 75 %B; 12.1-17 min, 75 %B. The injection volume
177 was 5 µL. Quantification was performed on an Agilent 6470 Triple Quadrupole MS equipped with a Jet
178 Stream ion source (Agilent Technologies, Santa Clara, CA) using positive multi reaction monitoring (MRM)
179 mode. The specific instrument parameters can be found in (Barnkob et al., 2019). The $[M+H]^+$ ions of the
180 derivatised analytes were used as precursor ions: vitamin D_3, 560.3 *m/z* and vitamin D_3-d_6, 566.3 *m/z*.
181 298.1 *m/z* were used as product ions for both the analogues. In addition a product ion scan method was set
182 up that used a collision energy of 10 and scanned from 150-600 m/z for the chosen precursors: $[M+H]^+$
183 560.3 *m/z*; $[M+Na]^+$ 582.3 *m/z*; $[M+NH4]^+$ 577.3 *m/z* ;$[M+H+NH4]^{2+}$ 289.2 *m/z*.
184 4 µl/min of vitamin D_3-d_6 were infused post-column through a tee using a KD Scientific syringe pump
185 (Holliston, MA, USA) with a 1 mL S.G.E gastight syringe during the chromatographic runs.
186 The whole sequence was run twice, once with 0 % formic acid in the mobile phase and then with 0.05
187 % formic acid. In both cases a run without injection was included.

188 The infusion chromatograms were visualised using Agilent MassHunter Qualitative Analysis (v.
189 B.07.00). Chromatograms were overlaid in regards to the RT of inherent vitamin D_3 in the samples if there
190 were small shifts in RT between runs.
191
192 **3. Results**
193
194 *3.1. Comparison of in-house reference results obtained using different assays*
195
196 At least one of our in-house reference materials is included when we extract samples and the
197 determined vitamin D is inserted into an x-chart. The results for eggs, cheese, salmon, and pork fat are
198 displayed in Table 1. From Table 1 it can be seen that the HPLC-UV-DAD and the LC-MS/MS method with
199 methylamine/formic acid in the eluent gave comparable results for cheese, salmon and pork fat while for
200 eggs differed significantly. Using the LC-ESI-MS/MS method with ammonium formate as additive
201 introduced the necessity to correct for recovery. When using an eluent with 2.5 mM ammonium formate as
202 the only additive the vitamin D_3 content was overestimated with around 30 % in-house egg yolk reference
203 samples.
204

205 **Table 1.** Content and recovery of vitamin D_3 in different in-house reference samples as determined by four different methods. The
206 content shown was calculated directly from the calibration curves. Contents marked with * indicate that the results had to be
207 corrected for recovery.

	Vitamin D_3 (ng/g)			
	HPLC-UV	LC-ESI-MS/MS		
	UV	Methylamine 0.1 % formic acid	Ammonium formate 0 % formic acid	Ammonium formate 0.05% formic acid
Egg ref	26.8 ± 2.0 (n=23)	27.7 ± 1.7 (n= 11)	35.4 ± 1.8 (n=15)*	29.3 ± 1.9 (n=6)
Salmon	154 ± 3 (n=5)	155 ± 7 (n=7)	143 ± 17 (n=4)	154 ± 8 (n=4)
Cheese	-	1.1 ± 0.1 (n=9)	1.1 ± 0.1 (n=23)	1.1 ± 0.1 (n=4)
Fat	-	3.6 ± 0.1 (n=4)	4.1 ± 0.7 (n=27)	4.1 ; 4.2 (n=2)
Recovery**	93 ± 7 (n=38)	82-97 (n=16)***	99 ± 9 (n=13)	-

208 * Determined from calibration curve - corrected for recovery before being reported
209 ** Except eggs
210 *** From (Burild, Frandsen, Poulsen, & Jakobsen, 2015)
211
212

213 *3.2. The effect of eluent additives on ion suppression*

214

215 *3.2.1. Effect of adding formic acid to an eluent already containing ammonium formate*

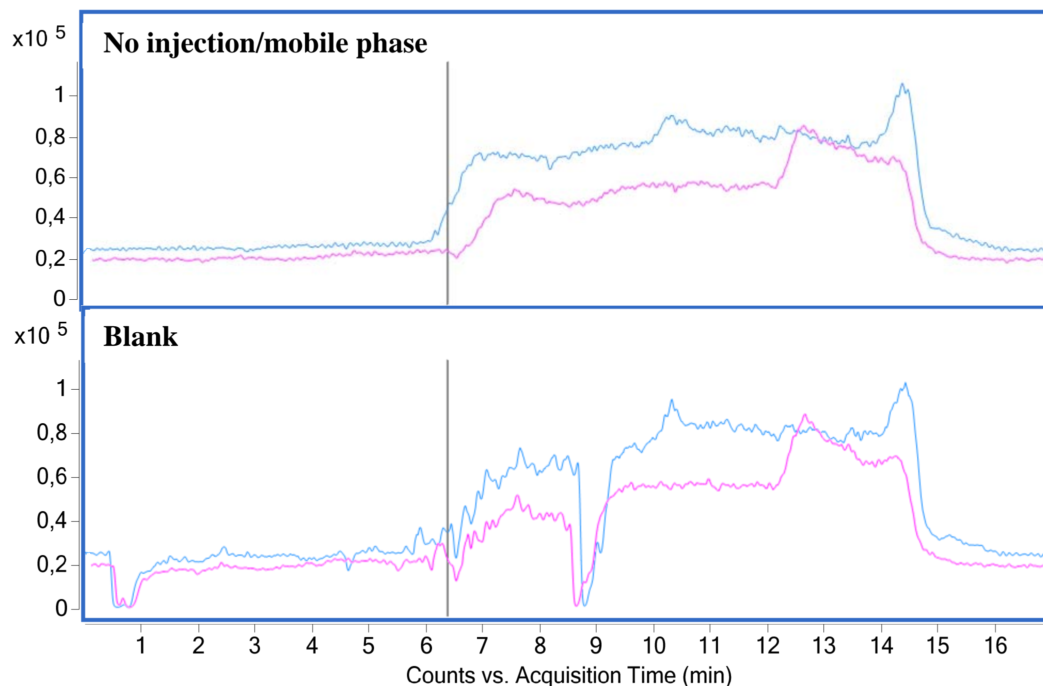
216 In the post-column infusion chromatogram obtained without injection (i.e. it shows the effect of the
217 mobile phase composition) an increase in the ionisation efficiency with increased percentage of methanol
218 was observed (see Figure 1); this has also been reported by others (Dams, Benijts, Günther, Lambert, &
219 Leenheer, 2002). The vertical black line in the full chromatograms shows the retention time of vitamin D_3;
220 vitamin D_3-d_6 eluted 0.019 minutes before. Using a combination of 2.5 mM ammonium formate and 0.05 %
221 formic acid as eluent additives (pink in Figure 1) decreased the ionisation efficiency and delayed the effect
222 of increasing the percentage of methanol compared to an eluent with 2.5 mM ammonium formate alone
223 (blue in Figure 1); resulting in the vitamin D_3 analogues eluted in an area with stable ionisation efficiency in
224 regard to the eluent. It should be noted that even though the slope at the elution time of vitamin D_3 looked
225 steep for the eluent without formic acid, the actual increase in signal intensity within the retention window
226 was around 0.45 x 10^4 which is minor compared to what we observed for ME in sample extracts (see Figure
227 2).

228 When comparing the infusion chromatogram of the mobile phase to the infusion chromatogram of the

229 blank it is seen that the sample clean-up itself introduces ion suppression (see Figure 1).

230

231



232

233 **Figure 1**. Post-column infusion chromatograms of vitamin D_3-d_6 ($[M+H]^+$ 566.3 *m/z* → 298.1 *m/z*) obtained without sample injection
234 i.e. mobile phase and of an extraction blank. A segmented linear gradient with 75% MeOH to 100 % was used. The vertical line
235 shows the retention time of vitamin D_3. The blue suppression pattern was obtained using an eluent with 2.6 mM ammonium
236 formate and 0 % formic acid. The pink was obtained using an eluent with 2.5 mM ammonium formate and 0.05 % formic acid (If
237 printed in black/white, pink will be darkest)**.**

238

239 Ion suppression chromatograms obtain from running sample extracts of salmon, cheese, egg yolk and

240 liver gave different ion suppression patterns but in common for all was a lot of suppression between 5-7

241 minutes (see Figure 2). Using a combination of 0.05 % formic acid and 2.5 Mm ammonium (pink in Figure 2)

242 decreased the ion suppression and thereby decreased the difference in the ionisation efficiency of the

243 vitamin D_3 analogous during injection of egg yolk, salmon and cheese extracts compared to the eluent with

244 2.5 mM ammonium formate alone (blue in Figure 2).

Somehow the matrix of the liver sample almost completely removed the effect of adding formic acid to the eluent making the two chromatograms almost the exact same; and the ion suppression was almost completely stable during the elution of the vitamin D_3 analogous, as can be seen in Figure 2.

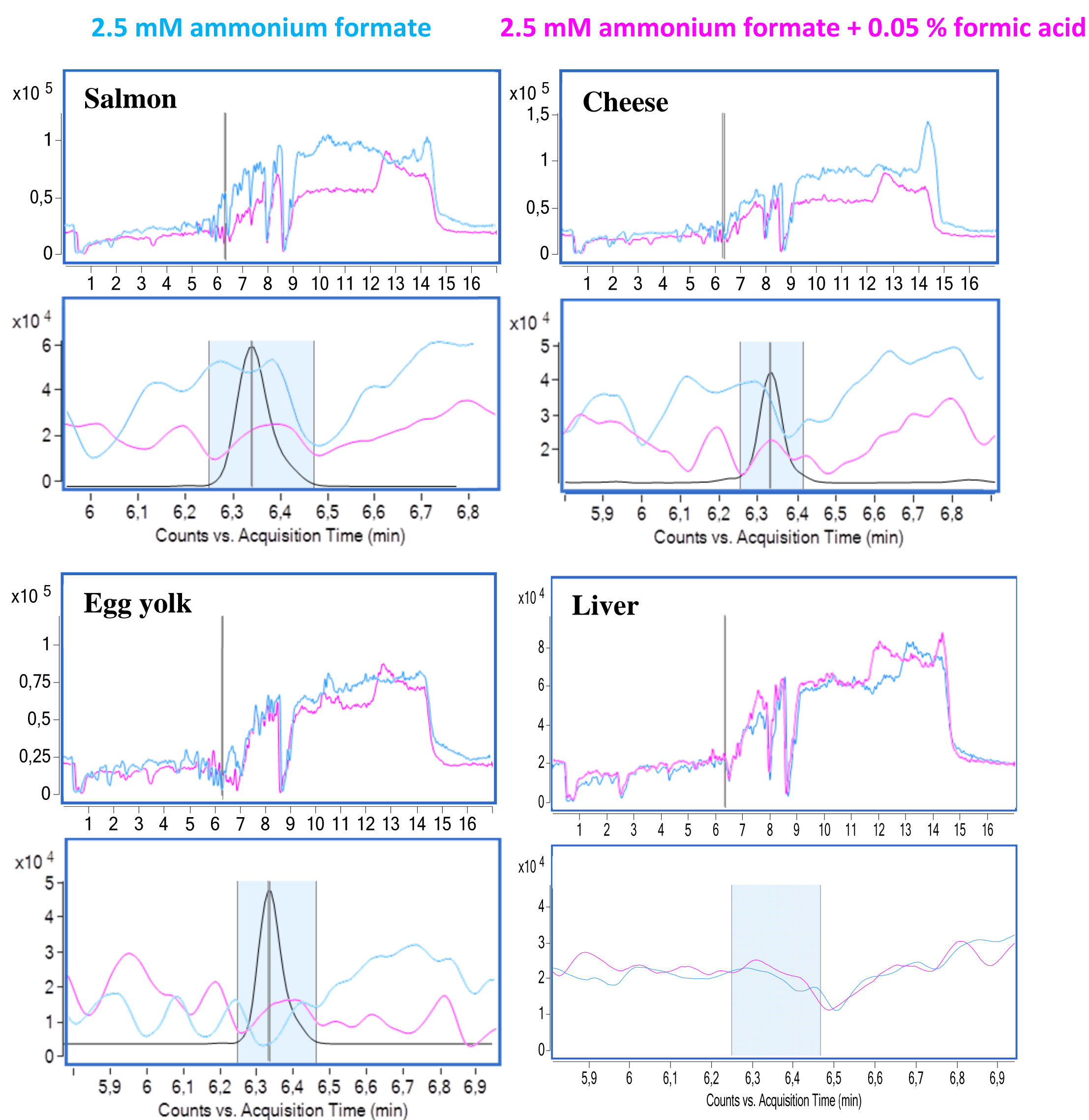


Figure 2. Post column infusion of *d6*-vitamin D_3 (566.3 *m/z* → 298.1 *m/z*) obtained during running of extracts of salmon, cheese, egg yolk and liver. A segmented linear gradient with 75% MeOH to 100 % was used. The chromatograms denoted with sample names are the full chromatograms while the chromatograms below are zoomed-in. The vertical line in the full chromatograms shows the retention time of vitamin D_3 while zoom-ins are overlaid with the vitamin D_3 peak (560.3 *m/z* → 298.1 *m/z*). The blue suppression pattern was obtained using an eluent with 2.5 mM ammoniumformate. The pink was obtained using an eluent with 2.5 mM ammonium formate and 0.05 % formic acid (If printed in black/white, pink will be darkest).

259 The ion suppression chromatograms obtained from injection of 1546a, pork loin from 2006, pork loin

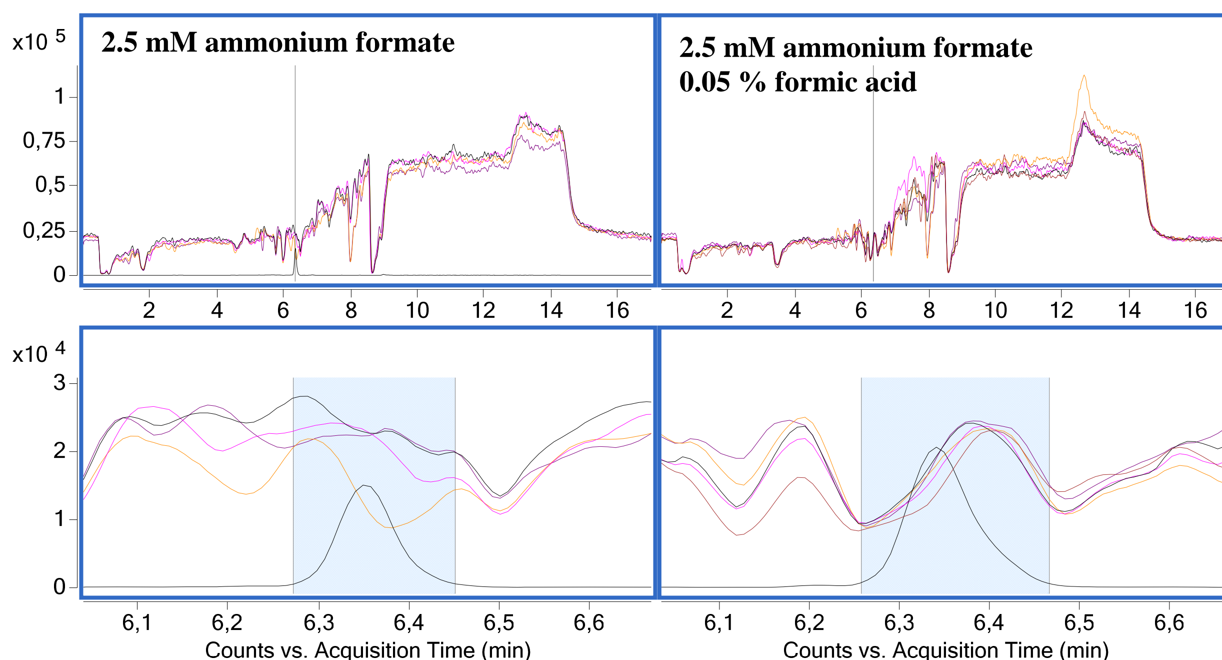
260 from 2016 and beef extracts were comparable when 2.5 mM ammonium formate was used alone (see

261 Figure 3); and at the RT of the vitamin D_3 analogous the ion suppression was relatively stable. However,

262 when adding 0.05 % formic acid to the eluent containing 2.5 mM ammonium formate the suppression

263 patterns became nearly identical in the retention window of the vitamin D_3 analogues (see Figure 3).

264



265

266 **Figure 3.** Post-column infusion chromatograms of vitamin D_3-d_6 (566.3 *m/z* → 298.1 *m/z*) during injection of extracts of Nist® SRM®
267 1546a meat homogenate (purple), pork loin (2006, orange), pork loin (2016, black), beef (pink) and pork fat (brown) using
268 ammonium formate (left) or ammonium formate/formic acid (right) as the eluent additive (the run of pork fat failed and was
269 therefore omitted from 2.5 mM ammonium formate alone). A segmented linear gradient with 75% MeOH to 100 % was used. The
270 vertical line in the upper chromatograms shows the retention time of vitamin D_3 while the lower chromatograms are overlaid with
271 the vitamin D_3 peak (560.3 *m/z* → 298.1 *m/z*). Above is the full chromatogram, below is a zoom-in on the retention window of
272 vitamin D_3.

273

274 *3.2.2. Effect of using methylamine in the eluent*

275 Only the blank, egg yolk, 1546a and pork loin (2006) were used to visualise the effect addition of

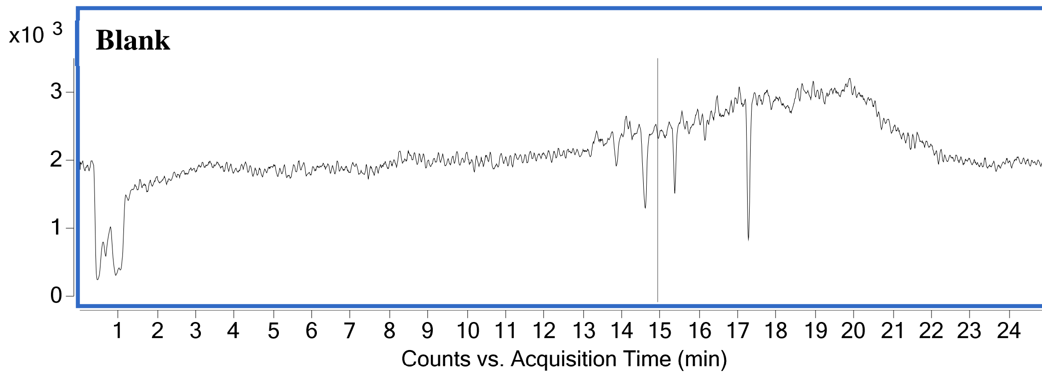
276 methylamine to the eluent has on the ion suppression. The methylamine adduct of vitamin D_3-d_6

277 ($[M+CH_3NH_3]^+$ 597.4 *m/z* → 298.1 *m/z*) was much less prone to ion suppression and only four peaks of

278 suppression was observed, in the infusion chromatogram of the blank extract, and non at the RT of vitamin

279 D_3 (compare to Figure 4 to Figure 1).

280

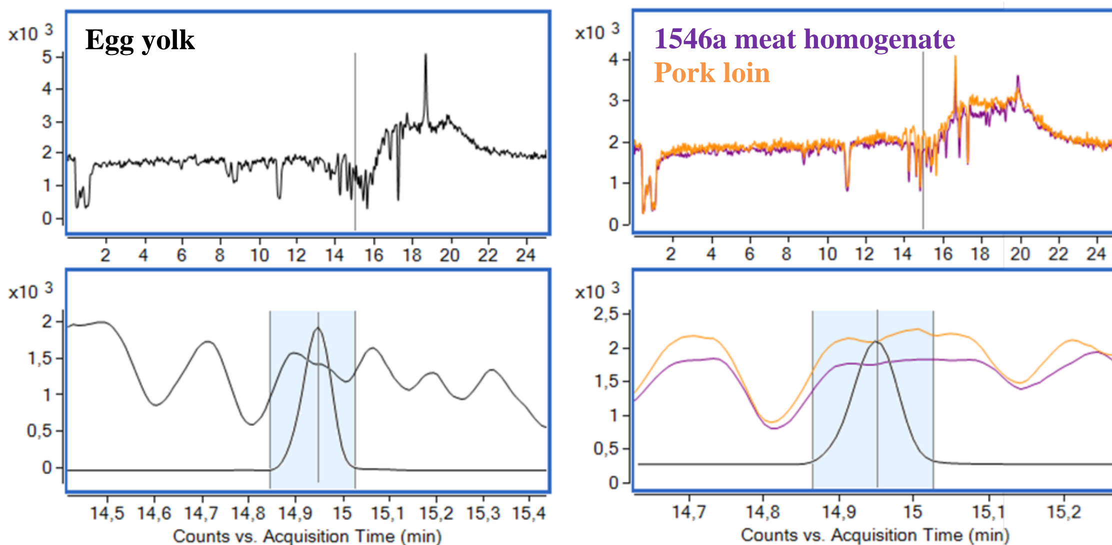


281

282 **Figure 4.** Post-column infusion chromatogram of vitamin D_3-d_6 methylamine adduct ($[M+H]^+$ 566.3 *m/z* → 298.1 *m/z*)
283 during run of a sample blank. A segmented linear gradient going from 60% MeOH to 100 % was used. The eluent
284 additives were 5 mM methylamine and 0.1 % formic acid.

285

286 As for the blank the ion suppressions patterns of the egg yolk, 1546a and pork loin (2006) extracts was
287 simpler when run with methylamine/formic acid in the eluent compared to an eluent with ammonium
288 formate (compare Figure 5 with Figure 2 and 3). However, there were still a lot of suppressions close to the
289 RT of the vitamin D_3 analogues but they eluted in a relatively steady area in between two suppression
290 regions as seen from the zoom-ins in Figure 5.



291

292 **Figure 5.** Post-column infusion chromatograms of the methylamine adduct of vitamin D_3-d_6 ($[M+CH_3NH_3]^+$ 597.4 *m/z* → 298.1 *m/z*)
293 obtained during run of extracts of egg yolk and 1546a meat homogenate (purple) and pork loin (2006, orange). The eluent
294 contained 5 mM methylamine and 0.1 % formic acid and a segmented linear gradient with 60% to 100 % MeOH was used.

295

296 *3.3. Effect of ammonium formate and formic acid on adduct formation*

297 The proton, sodium and ammonium adducts were selected for product ion scan. Only the protonated
298 adduct produced a product ion with significant abundance, the other adducts remained intact during
299 collision. When no additives were added to the eluent the most abundant ion was the sodium adduct (see
300 Table 2). Adding ammonium formate alone increased the abundance of the protonated adduct significantly
301 and its product ion was increased more than 40 times; the ammonium adducts were reduced by a factor of
302 9-15 while the sodium adduct was no affected. Adding formic acid alone decreased the abundance of all
303 the ions by at least 50 %. Adding both ammonium formate and formic acid to the eluent maintained the
304 effect of formic acid on the sodium and ammonium adducts while the effect of ammonium formate on the
305 protonated adducts and its product ion was significantly reduced.

306 **Table 2.** The abundance of the proton, sodium and ammonium adduct of vitamin D_3 at different combinations of ammonium
307 formate and formic acid in the eluent. Determined using product ion scan from 150 *m/z* to 600 *m/z,* collision energy 10. Only the
308 protonated ion gave rise to a product ions with high abundance, the other adducts stayed intact during collision.

Eluent additive (mM)		Abundance (x 10^4)				
Ammonium Formate	Formic acid	[M+H]+→298.1	$[M+H]^+$	$[M+Na]^+$	$[M+NH4]^+$	$[M+H+NH4]^{2+}$
0 mM	0 %	2.0	2.4	14	2.3	3.3
2,5 mM	0 %	88	11	17	0.15	0.37
2,5 mM	0,05 %	9.1	2.8	5.7	1.6	1.1
0 mM	0,05 %	1.0	1.1	5.7	1.4	2.1

309

310

311 **4. Discussion**

312 When we changed our eluent additive from methylamine/formic acid to ammonium formate in our
313 LC-ESI-MS/MS method it performed well for cheese and cream samples, giving the expected results in in-
314 house references and recovery samples. However, for egg yolk, and sometimes for pork and salmon the
315 determined vitamin D_3 in the in-house references and the recovery samples were biased and therefore the
316 necessity to correct for recovery was introduced. If not corrected for recovery the vitamin D_3 content in egg
317 yolk would be overestimated; this was quite surprising as we used vitamin D_3-d_6 as internal standard; and

318 although it elutes 0.019 minutes before PTAD-vitamin D_3 we expected the ion suppression to be
319 comparable to PTAD-vitamin D_3 but a recovery of 135 % for egg yolk could not be explained by variation
320 between different methods. In addition the method was not completely robust as once in a while there
321 where runs that gave unexpected results and therefore needed to be run again. We tried to adjust the
322 gradient including trying the exact same gradient as used in the methylamine method although without any
323 change observed. We ruled out the instrument by running the methylamine method on our new
324 instrument and obtained similar results as earlier on our old MS, however remnants of methylamine
325 remained in the HPLC equipment and decreased the signal intensity for vitamin K by approximately 50 % in
326 subsequent runs. Not wanting to go back to using methylamine we were determined to solve the reason
327 behind our troubles with the eluent additives. We therefore looked into the difference between using
328 methylamine/formic acid and ammonium formate as additive. The pH of the 5 mM methylamine and 0.1 %
329 formic acid measured in water was 3.2 while the pH of 2.5 mM ammonium formate in water was measured
330 to be 6.1. Formic acid has a pKa of 3.75 while ammonium has a pKa of 9.25, ammonium formate will
331 therefore not function as a buffer at pH 6.1 (Konermann, 2017)**.** Adding 0.05 % formic acid in addition to
332 the 2.5 mM ammonium formate in water lowered the pH to 3.2 that was within the buffering capacity.
333 During ESI water is oxidized whereby the pH can be lowered by up to 4 pH units. However the pH is most
334 affected in systems that are unbuffered and around neutral pH (Van Berkel, Zhou, & Aronson, 1997). When
335 references were analysed using a combination of ammonium formate and formic acid in the eluent the
336 quantitation of vitamin D_3 were closer to our old methods; however the method was still not robust. We
337 hypothesised that ME was the cause of our problems but we were completely blindfolded in what caused
338 the ion suppression. We therefore used continuous post-column infusion of PTAD-vitamin D_3-d_6 to visualise
339 the ion suppression of different matrices and how it was affected by eluent additives. To our knowledge
340 we are the first to report that post-column infusion experiments can be performed by using SIL IS when a
341 blank matrix is not available. Although there might be a small difference in how ME affects SIL IS and their
342 analogous (Berg & Strand, 2011) it is the closest we come to the physical and chemical likeness of the

343 analyte. One might argue that the cost will be too high, which might be the case in some situations;
344 however, we have vitamin D_3-d_6 in stock as we use it for routine analysis and the cost of the 50 mL of
345 solution prepared (much more than needed) for this experiment has a value of less than 12 Euro.
346 We were not able to explain the big difference we see in recovery between the eluent with
347 ammonium formate and ammonium formate/formic acid from the post-column infusion chromatograms.
348 We did however visualise that ion suppression due to ME were quite pronounced for most matrices
349 especially around the retention time of the PTAD-vitamin D_3 analogous. When comparing the infusion
350 chromatograms of the different food samples (Figure 2) it is clear that a method for determining vitamin D
351 in food is not *per se* universal and MEs have to be assessed in all types of food samples one might want to
352 analyse. However, the ion suppression observed in pork meat (from 2006 and 2016), pork fat, 1546a meat
353 homogenate and beef meat was comparable therefore it could be possible to make a full validation on only
354 one of those matrices. The sample clean-up itself also introduces some ion suppression (see Figure 1). The
355 origin is probably additives from plastic as most of the clean-up steps involve single use plastic tubes as well
356 as plastic Pasteur pipettes and pipette tips; however they first start eluting after 6.5 minutes right after
357 PTAD-vitamin D_3 is fully eluted. Adding 0.05 % formic acid to the eluent decreased the ion suppression
358 (pink chromatogram in Figure 1 and 2) and thereby the difference in the ionisation efficiency of the vitamin
359 D_3 analogous was decreased whereby the accuracy was increased as seen from Table 1; however adding
360 formic acid significantly decreased the abundance of the product ion used for quantification (see Table 2).
361 The methylamine adduct was less prone to ion suppression and only four peaks of suppression were
362 observed in the blank and non at the RT of PTAD- vitamin D_3, see Figure 4 and 5. The methylamine method
363 had a longer runtime and a slightly different gradient but most likely this did not account for the reduced
364 ion suppression. That PTAD-vitamin D_3 elutes in a relative stable area in regards to ion suppression in the
365 methylamine method seems to be a case of good luck as even though the methylamine adduct is less prone
366 to ion suppression there are still a lot present close to the retention window of the PTAD-vitamin D_3
367 analogues (Figure 5).

368 Adding ammonium formate to the eluent increased the signal of PTAD-vitamin D_3 with more than a
369 factor 40 (see section 3.3) while the ammonium adducts were decreased with a factor 9-15; however, the
370 robustness and the accuracy of this methods was however not good enough, adding formic acid to the
371 eluent helped with the accuracy but not the robustness. The lack of robustness was most likely caused by
372 day to day variations that affected the ion suppression. To get the accuracy to be less affected by varying
373 ME would therefore require a more extensive sample clean-up or the use of a ^{13}C labelled SIL IS instead; ^{13}C
374 labelled SIL ISs have been shown to be superior to ^{2}H labelled as they co-elute with their corresponding
375 analytes whereby ME is significantly decreased (Berg & Strand, 2011), they are however expensive and not
376 always available. For vitamin D_3 the $^{13}C_5$ labelled SIL IS is more than twice as expensive as the deuterated
377 however the cost of extra technician hours, equipment and solvents for a more extensive sample clean-up
378 is expected to be more costly. The accuracy obtained with our old HPLC-DAD-UV method relied on
379 extensive clean-up and preparatory HPLC until the full absorption spectra of vitamin D_3/vitamin D_2 were
380 shown to be completely pure when measured using a diode array detector (DAD) (Jakobsen et al., 2004);
381 this procedure protected against interferences that would not be observed if the peak were only recorded
382 using one wavelength as reported by (William C. Byrdwell, 2009)**.**
383
384 **4. Conclusion**

385

386 Post-column infusion experiments can be performed by using deuterated SIL IS when a blank matrix is
387 not available; and by using post-column infusion with PTAD-vitamin D_3-d_6 we were able to visualise the
388 effect different eluent additives have on ion suppression in extractions of different matrices. With a sample
389 preparation consisting of saponification, LLE, SPE and derivatisation with PTAD we observed an enormous
390 amount of ion suppression. From our results it was clear that due to its earlier RT deuterated SIL IS cannot
391 fully eliminate ME when analysing vitamin D_3 in most food matrices. To get the accuracy to be completely
392 independent of ME would require a more extensive sample clean-up or use a ^{13}C labelled SIL IS instead. For

vitamin D_3 the $^{13}C_5$ labelled SIL IS is more than twice as expensive as the deuterated however the cost of extra technician hours, equipment and solvents for a more extensive sample clean-up is expected to be more costly.

5. References

Barnkob, L. L., Petersen, P. M., Nielsen, J. P., & Jakobsen, J. (2019). Vitamin D enhanced pork from pigs exposed to artificial UVB light in indoor facilities. *European Food Research and Technology*, *245*(2), 411–418. http://doi.org/10.1007/s00217-018-3173-6

Berg, T., & Strand, D. H. (2011). 13C labelled internal standards-A solution to minimize ion suppression effects in liquid chromatography-tandem mass spectrometry analyses of drugs in biological samples? *Journal of Chromatography A*, *1218*(52), 9366–9374. http://doi.org/10.1016/j.chroma.2011.10.081

Bilodeau, L., Dufresne, G., Deeks, J., Clément, G., Bertrand, J., Turcotte, S., … Fouquet, A. (2011). Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC UV-DAD and LC–MS/MS. *Journal of Food Composition and Analysis*, *24*(3), 441–448. http://doi.org/10.1016/j.jfca.2010.08.002

Bonfiglio, R., Bonfiglio, R., King, R., King, R., Olah, T., Olah, T., … Merkle, K. (1999). The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Communications in Mass Spectrometry : RCM*, *13*(12), 1175–1185. http://doi.org/10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0

Buhrman, D. L., Price, P. I., & Rudewicz, P. J. (1996). Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: A study of ion suppression. *Journal of the American Society for Mass Spectrometry*, *7*(11), 1099–1105. http://doi.org/10.1016/S1044-0305(96)00072-4

Burild, A., Frandsen, H. L., Poulsen, M., & Jakobsen, J. (2014). Quantification of physiological levels of vitamin D3 and 25-hydroxyvitamin D3 in porcine fat and liver in subgram sample sizes. *Journal of Separation Science*, *37*(19), 2659–63. http://doi.org/10.1002/jssc.201400548

Burild, A., Frandsen, H. L., Poulsen, M., & Jakobsen, J. (2015). Tissue content of vitamin D3 and 25-hydroxy vitamin D3 in minipigs after cutaneous synthesis, supplementation and deprivation of vitamin D3. *Steroids*, *98*, 72–9. http://doi.org/10.1016/j.steroids.2015.02.017

Byrdwell, W. C. (2009). Comparison of Analysis of Vitamin D3 in Foods Using Ultraviolet and Mass Spectrometric Detection. *Journal of Agricultural and Food Chemistry*, *57*(6), 2135–2146. http://doi.org/10.1021/jf803398u

Byrdwell, W. C., DeVries, J., Exler, J., Harnly, J. M., Holden, J. M., Holick, M. F., … Wolf, W. R. (2008). Analyzing vitamin D in foods and supplements: methodologic challenges. *The American Journal of Clinical Nutrition*, *88*(2), 554S–557S. http://doi.org/10.1093/ajcn/88.2.554S

Byrdwell, W. C., Horst, R. L., Phillips, K. M., Holden, J. M., Patterson, K. Y., Harnly, J. M., & Exler, J. (2013). Vitamin D levels in fish and shellfish determined by liquid chromatography with ultraviolet detection

430 and mass spectrometry. *Journal of Food Composition and Analysis*, *30*(2), 109–119.
431 http://doi.org/10.1016/j.jfca.2013.01.005

432 Dams, R., Benijts, T., Günther, W., Lambert, W., & Leenheer, A. De. (2002). Influence of the eluent
433 composition on the ionization efficiency for morphine of pneumatically assisted electrospray,
434 atmospheric-pressure chemical ionization and sonic spray. *Rapid Communications in Mass*
435 *Spectrometry*, *16*(11), 1072–1077. http://doi.org/10.1002/rcm.683

436 Dimartino, G. (2009). Simultaneous Determination of Cholecalciferol (Vitamin D3) and Ergocalciferol
437 (Vitamin D2) in Foods by Selected Reaction Monitoring. *Journal of AOAC International*, *92*(2), 511–
438 517.

439 Furey, A., Moriarty, M., Bane, V., Kinsella, B., & Lehane, M. (2013). Ion supression; A critical review on
440 causes , evaluation , prevention and applications. *Talanta*, *115*, 104–122.
441 http://doi.org/10.1016/j.talanta.2013.03.048

442 Gilliland, D. L., Black, C. K., Denison, J. E., Seipelt, C. T., & Dowell, D. (2012). Simultaneous Determination of
443 Vitamins D 2 and D 3 by LC-MS/MS in Infant Formula and Adult Nutritionals: First Action 2011.13.
444 *Journal of AOAC International*, *95*(3), 583–587. http://doi.org/10.5740/jaoacint.CS2011_13

445 Gomes, F. P., Shaw, P. N., Whitfield, K., & Hewavitharana, A. K. (2015). Simultaneous quantitative analysis
446 of eight vitamin D analogues in milk using liquid chromatography–tandem mass spectrometry.
447 *Analytica Chimica Acta*, *891*, 211–220. http://doi.org/10.1016/J.ACA.2015.08.017

448 Heudi, O., Trisconi, M.-J., & Blake, C.-J. (2004). Simultaneous quantification of Vitamins A, D3 and E in
449 fortified infant formulae by liquid chromatography-mass spectrometry. *Journal of Chromatography A*,
450 *1022*, 115–123. http://doi.org/10.1016/j.chroma.2003.09.062

451 Höller, U., Quintana, A. P., Gössl, R., Olszewski, K., Riss, G., Schattner, A., & Nunes, C. S. (2010). Rapid
452 determination of 25-hydroxy vitamin D3 in swine tissue using an isotope dilution HPLC-MS assay.
453 *Journal of Chromatography B*, *878*(13–14), 963–968. http://doi.org/10.1016/J.JCHROMB.2010.02.026

454 Huang, M., Winters, D., Sullivan, D., & Dowell, D. (2012). Application of Ultra-High-Performance Liquid
455 Chromatography/ Tandem Mass Spectrometry for the Measurement of Vitamin D in Infant Formula
456 and Adult/Pediatric Nutritional Formula: First Action 2011.11. *Journal of AOAC International*, *95*(2),
457 319–321. http://doi.org/10.5740/jaoacint.CS2011_11

458 Jakobsen, J., Clausen, I., Leth, T., & Ovesen, L. (2004). A new method for the determination of vitamin D3
459 and 25-hydroxyvitamin D3 in meat. *Journal of Food Composition and Analysis*, *17*, 777–787.
460 http://doi.org/10.1016/j.jfca.2003.10.012

461 Jakobsen, J., Maribo, H., Bysted, A., Sommer, H. M., & Hels, O. (2007). 25-Hydroxyvitamin D3 affects
462 vitamin D status similar to vitamin D3 in pigs–but the meat produced has a lower content of vitamin
463 D. *British Journal of Nutrition*, *98*(05), 908–913. http://doi.org/10.1017/S0007114507756933

464 Jakobsen, J., & Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *Journal of Food*
465 *Composition and Analysis*, *22*(5), 472–478. http://doi.org/10.1016/j.jfca.2009.01.010

466 Konermann, L. (2017). Addressing a Common Misconception: Ammonium Acetate as Neutral pH “Buffer”
467 for Native Electrospray Mass Spectrometry. *Journal of The American Society for Mass Spectrometry*,
468 *28*(9), 1827–1835. http://doi.org/10.1007/s13361-017-1739-3

469 Lietzow, J., Kluge, H., Brandsch, C., Seeburg, N., Hirche, F., Glomb, M., & Stangl, G. I. (2012). Effect of short-

470 term UVB exposure on vitamin D concentration of eggs and vitamin D status of laying hens. *Journal of*
471 *Agricultural and Food Chemistry*, *60*(3), 799–804. http://doi.org/10.1021/jf204273n

472 Lipkie, T. E., Janasch, A., Cooper, B. R., Hohman, E. E., Weaver, C. M., & Ferruzzi, M. G. (2013).
473 Quantification of vitamin D and 25-hydroxyvitamin D in soft tissues by liquid chromatography–tandem
474 mass spectrometry. *Journal of Chromatography B*, *932*, 6–11.
475 http://doi.org/10.1016/J.JCHROMB.2013.05.029

476 Matuszewski, B. K. (2006). Standard line slopes as a measure of a relative matrix effect in quantitative
477 HPLC–MS bioanalysis. *Journal of Chromatography B*, *830*(2), 293–300.
478 http://doi.org/10.1016/J.JCHROMB.2005.11.009

479 Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix
480 effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, *75*(13),
481 3019–3030. http://doi.org/10.1021/ac020361s

482 Roseland, J. M., Patterson, K. Y., Andrews, K. W., Phillips, K. M., Phillips, M. M., Pehrsson, P. R., … Taylor, C.
483 L. (2016). Interlaboratory Trial for Measurement of Vitamin D and 25-Hydroxyvitamin D [25(OH)D] in
484 Foods and a Dietary Supplement Using Liquid Chromatography–Mass Spectrometry. *Journal of*
485 *Agricultural and Food Chemistry*, *64*(16), 3167–3175. http://doi.org/10.1021/acs.jafc.5b05016

486 Schadt, H. S., Gössl, R., Seibel, N., & Aebischer, C.-P. (2012). Quantification of Vitamin D3 in Feed, Food, and
487 Pharmaceuticals Using High-Performance Liquid Chromatography/Tandem Mass Spectrometry.
488 *Journal of AOAC International*, *95*(5), 1487–1494. http://doi.org/10.5740/jaoacint.11-512

489 Stevens, J., & Dowell, D. (2012). Determination of Vitamins D2 and D3 in Infant Formula and Adult
490 Nutritionals by Ultra-Pressure Liquid Chromatography with Tandem Mass Spectrometry Detection
491 (UPLC-MS/MS): First Action 2011.12. *Journal of AOAC International*, *95*(3), 577–582.
492 http://doi.org/10.5740/jaoacint.CS2011_12

493 Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., & Greenfield, H. (2013). Vitamin D and 25-
494 hydroxyvitamin D determination in meats by LC–IT-MS. *Food Chemistry*, *138*(2–3), 1042–1047.
495 http://doi.org/10.1016/J.FOODCHEM.2012.08.041

496 Trenerry, V. G., Plozza, T., Caridi, D., & Murphy, S. (2011). The determination of vitamin D3 in bovine milk
497 by liquid chromatography mass spectrometry. *Food Chemistry*, *125*(4), 1314–1319.
498 http://doi.org/10.1016/j.foodchem.2010.09.097

499 Trufelli, H., Palma, P., Famiglini, G., & Cappiello, A. (2011). An overview of matrix effects in liquid
500 chromatography-mass spectrometry. *Mass Spectrometry Reviews*, *30*(3), 491–509.
501 http://doi.org/10.1002/mas.20298

502 Van Berkel, G. J., Zhou, F., & Aronson, J. T. (1997). Changes in bulk solution pH caused by the inherent
503 controlled-current electrolytic process of an electrospray ion source. *International Journal of Mass*
504 *Spectrometry and Ion Processes*, *162*(1–3), 55–67. http://doi.org/10.1016/S0168-1176(96)04476-X

505 Wang, S., Cyronak, M., & Yang, E. (2007). Does a stable isotopically labeled internal standard always correct
506 analyte response?. A matrix effect study on a LC/MS/MS method for the determination of carvedilol
507 enantiomers in human plasma. *Journal of Pharmaceutical and Biomedical Analysis*, *43*(2), 701–707.
508 http://doi.org/10.1016/j.jpba.2006.08.010

509 Wieling, J. (2006). LC-MS-MS experiences with internal standards. *Chromatographia*, *55*(S1), S107–S113.
510 http://doi.org/10.1007/bf02493365

511 Wu, J., Wiegand, R., Lorusso, P., & Li, J. (2013). A stable isotope-labeled internal standard is essential for
512 correcting for the interindividual variability in the recovery of lapatinib from cancer patient plasma in
513 quantitative LC-MS/MS analysis. *Journal of Chromatography B*, *941*, 100–108.
514 http://doi.org/10.1016/j.jchromb.2013.10.011

515

National Food Institute
Technical University of Denmark

Kemitorvet
Building 202
2800 Kgs Lyngby
Tel. +45 35 88 7

www.food.dtu.dk