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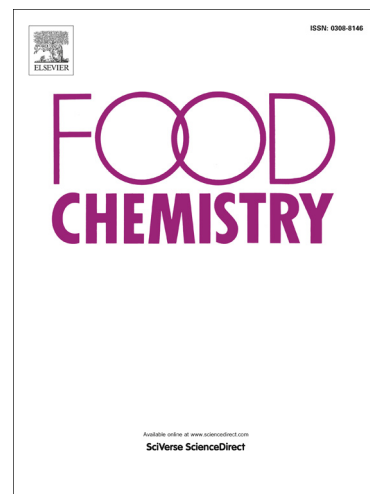
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Investigation of the effect of UV-LED exposure conditions on the production of vitamin D in pig skin.

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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 D₃ production. The maximum dose of 20 kJ/m^2 produced 3.5-4 μg vitamin D₃/cm² pig skin. Vitamin D₃ 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.

1. Introduction

The recommended human dietary intake of vitamin D is in the range 10-20 $\mu\text{g}/\text{day}$ (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/100g; fatty fish such as salmon, eel and mackerel contain 8-30 $\mu\text{g}/100\text{g}$, lean fish such as halibut, sole and tuna contain 3-9 $\mu\text{g}/100\text{g}$; while meat and dairy products contain less than 1 $\mu\text{g}/100\text{g}$ (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, Sommmmer, & Jakobsen, 2016).

It is possible to convert 7-dehydrocholesterol (7-DHC) to vitamin D₃ 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 D₃ (preD₃), which isomerises to form vitamin D₃ (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 D₃ and vitamin D₂, respectively. The principle is approved for use in the production of vitamin D₂ - enriched baker's yeast in the United States (Food and Drug Administration, 2012) and in the European Union (EFSA NDA Panel, 2014). Exposure of 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 D₃ 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 1x1 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 degrees). 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 Figure 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.

2.3. Experimental design

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

The objective was to determine how wavelength, dose and total irradiation influence vitamin D₃ production when pig skin is exposed to UV. In all exposures, the distance between the LED's and the sample of pig skin (1 cm²) 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, Figure S1).

2.3.1. Effect of wavelength on the production of vitamin D₃ in pig skin

The effect of wavelength on the production of vitamin D₃ was tested at two different doses; namely 300±3 J/m² and 7,000±3 J/m². The total irradiation emitted at these wavelengths varied between 8.2-12.4 W/m² and 2-23 W/m², respectively. The corresponding range in exposure time was 24-37 sec and 304-3,500 sec, respectively. The precision of the exposure time was estimated to ±0.5 sec to cover the experimentalist's response time to the timer. The LEDs were operated in constant current mode in the region between 100-600 mA (see SOM, Section S2, Table S1).

2.3.2. Effect of dose on the production of vitamin D₃ in pig skin

The LED, with a central wavelength of 296 nm, was used to determine the effect of 6 doses on the vitamin D₃ production in pig skin. The delivered doses were 207 J/m², 1,008 J/m², 2001 J/m², 6,002 J/m², 10,004 J/m² and 20,007 J/m². The exposure time was varied between 14 seconds and 22.50 minutes (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/m².

2.3.3. Effect of total irradiation and exposure time on the production of vitamin D₃ in pig skin

The total irradiation emitted by the LEDs was varied between 0.1-43 W/m² 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/m² (see SOM, Section S2, Table S3).

2.4. Analysis of vitamin D₃ and 7-DHC

2.4.1. Chemicals

The standards used were vitamin D₃ and vitamin D₂ 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 D₃ and D₂ assessed as 18,300 M⁻¹cm⁻¹ and 19,400 M⁻¹cm⁻¹, respectively (Norman, 1979) and for 7-DHC at 281 nm: 11,959 M⁻¹cm⁻¹. The value for 7-DHC was obtained from the designated vitamin D₂ equivalent, ergosterol (Sternberg, Stillo, & Schwendeman, 1960).

2.4.2. Procedure

The content of vitamin D₃ 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 cm² were thawed prior to analysis, and vitamin D₂ 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 D₂ and vitamin D₃ 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 D₂ and vitamin D₃ was performed on two C18 columns (VYDAC® 201TP, 5 µm, 250x4.6 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 D₂ and vitamin D₃, and 281 nm for 7-DHC. Vitamin D₂ was used as internal standard for vitamin D₃, whereas 7-DHC was quantified by use of the

external standard. The recovery of vitamin D₃ and 7-DHC were >90%. For vitamin D₃, the limit of quantification (LOQ) was 0.003 µg/cm² 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 D₃ as a function of wavelength

The content of vitamin D₃ in the pig skin after a UV dose of 300 J/m² and 7,000 J/m² was delivered, is displayed in Figure 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 D₃ content. Negligible amounts of vitamin D₃ were produced at 318 nm, while no vitamin D₃ 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 D₃ as a function of dose

At the optimum wavelength, 296 nm, the content of vitamin D₃ was determined at six different doses, and is displayed in Figure 3. The best fitted curve was a logarithmic curve ($y=0.6302 \text{ LN}(x) - 2.9049$) 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 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 D₃ has not been reached, and higher doses would give a higher content of vitamin D₃ in the pig skin. The content of 7-DHC in unexposed skin samples was determined to be $79 \pm 6 \mu\text{g}/\text{cm}^2$ ($n=3$). The highest obtained content of vitamin D₃ in the pig skin was between $3.5\text{-}4 \mu\text{g}/\text{cm}^2$, 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 $10,000\text{-}300,000 \text{ J}/\text{m}^2$ 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 $300,000 \text{ J}/\text{m}^2$. At this point approx. 70% of the initial 7-DHC had been converted to preD₃ (MacLaughlin et al., 1982). Furthermore, Takada et al. (1979) exposed rat skin with doses of $1530 \text{ J}/\text{m}^2$, $3,060 \text{ J}/\text{m}^2$, $6,120 \text{ J}/\text{m}^2$, $9,180 \text{ J}/\text{m}^2$ and $12,240$

J/m² (using a UV lamp, 280-310 nm). The amount of vitamin D₃ 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 0-4,500 J/m² using a wavelength of 300 nm. In the first case the vitamin D₃ 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 3,000 J/m², where it reached a plateau, and stayed constant up to 4,500 J/m², 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 D₃, and at some point a plateau will be reached thereby following a logarithmic pattern.

3.3. Production of vitamin D₃ 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 D₃ 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.

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 D₃.

3.4. Application in food production

The maximum achieved content of vitamin D₃ was 3.5-4 µg/cm² pig skin, and a content of vitamin D₃ in pig skin of 0.5 µg/cm² can be achieved with UV-LEDs by exposure for seven seconds.

Vitamin D₃ 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 D₃/100g would only require a content of 0.15 µg/cm² pig skin, assuming a skin surface area of 200 cm² 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 preD₃ are tachysterol₃ and lumisterol₃ (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). Lumisterol₃ has no affinity, and tachysterol₃ has a very low affinity for DBP which is why its presence in food will not influence the transport of vitamin D₃ in the circulation (Holick, 1981). According to an EFSA opinion on vitamin D₂ enriched foods, it is not necessary to include tachysterol₂ 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 tachysterol₃ 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 D₃.

4. Conclusion

The optimal wavelength for the production of vitamin D₃ 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 D₃ in pig skin follows a logarithmic curve. The maximum applied dose of 20 kJ/m² resulted in a vitamin D₃ content of 3.5-4 µg/cm².

An increase in content of vitamin D₃ 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 D₃.

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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

^aRange (± 3 sd) were estimated based on the Gaussian fit of the spectral distribution.

Table 2. References for investigation of optimum and no production of vitamin D.

Information on sample type and full width half maximum (FWHM).

Sample type	Optimum (nm)	No production (nm)	FWHM (nm)	Reference (Year)
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, Hirooka, & Yasumura, 1976)
Human skin	297	>320	3 or 5	(MacLaughlin et al., 1982)
<i>In vitro</i> human skin models	302	315	5	(Lehmann et al., 2001)
<i>In vitro</i> human skin models	302	-	5	(Lehmann et al., 2007)
7-dehydrocholesterol	295	315	1.7	(Olds et al., 2010)

Table 3. The content of vitamin D₃ after exposure to a dose of 300J/m² (295-302 J/m²) at 292, 296 and 300 nm at five different levels of total irradiation (0.1-43 W/m²).

Wavelength (nm)	Total irradiation (W/m ²)	Vitamin D ₃ ^a		<i>P</i> -value ^b
		(µg /cm ²)		
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	

^aEach exposure was repeated twice

^b*P*-values from one-way ANOVA, testing for no differences between total irradiation

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LEGENDS

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

Figure 2. Content of vitamin D₃ in pig skin as function of wavelength after a delivered dose of a) 300 J/m² (281-310 nm) and b) 7,000 J/m² (281-336 nm).

Figure 3. Production of vitamin D₃ in pig skin at different delivered doses of UVB at 296 nm.

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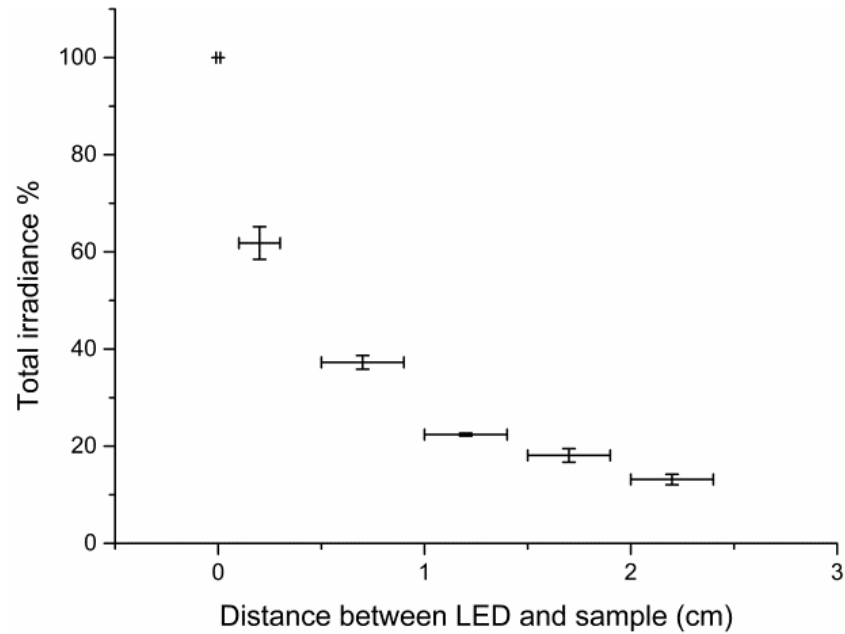


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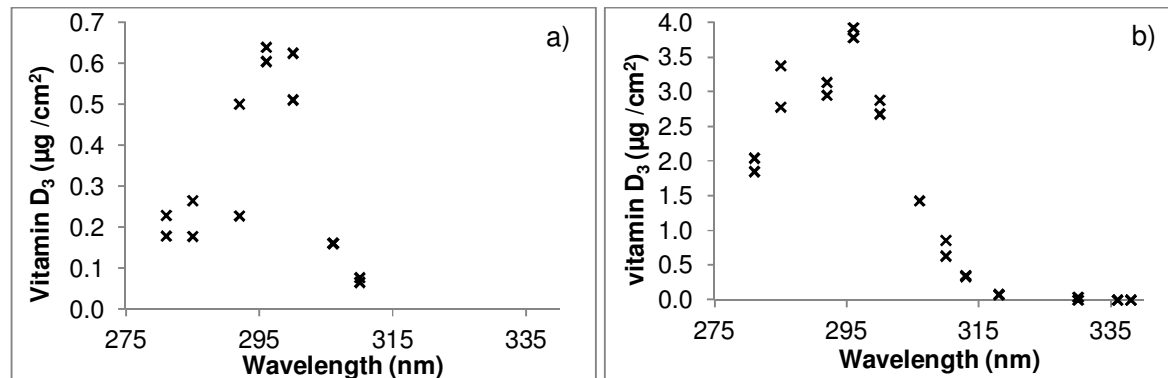


Figure 2. Content of vitamin D₃ in pig skin as function of wavelength after a delivered dose of a) 300 J/m² (281-310 nm) and b) 7,000 J/m² (281-336 nm).

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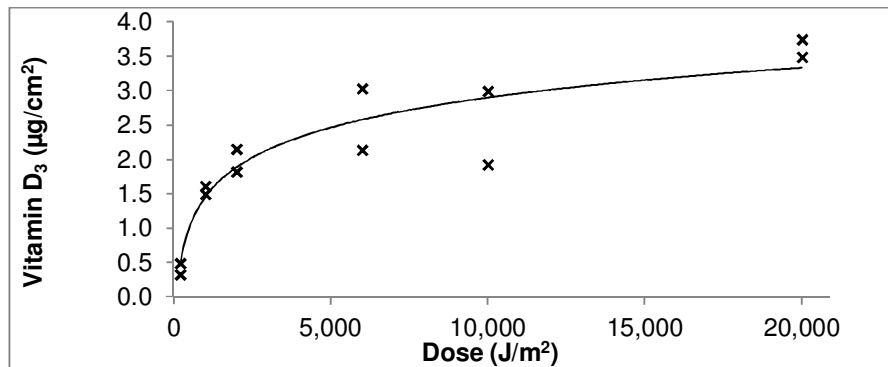


Figure 3. Production of vitamin D₃ in pig skin at different delivered doses of UVB at 296 nm.

Highlights

- ◆ Light-emitting diodes for production of vitamin D
- ◆ Vitamin D production dependent on dose of exposure
- ◆ Vitamin D bio-fortified pork products

ACCEPTED MANUSCRIPT