

## Exposure of vitamins to UVB and UVA radiation generates singlet oxygen

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Deleterious effects of UV radiation in tissue are usually attributed to different mechanisms. Absorption of UVB radiation in cell constituents like DNA causes photochemical reactions. Absorption of UVA radiation in endogenous photosensitizers like vitamins generates singlet oxygen *via* photosensitized reactions. We investigated two further mechanisms that might be involved in UV mediated cell tissue damage. Firstly, UVB radiation and vitamins also generate singlet oxygen. Secondly, UVB radiation may change the chemical structure of vitamins that may change the role of such endogenous photosensitizers in UVA mediated mechanisms. Vitamins were irradiated in solution using monochromatic UVB (308 nm) or UVA (330, 355, or 370 nm) radiation. Singlet oxygen was directly detected and quantified by its luminescence at 1270 nm. All investigated molecules generated singlet oxygen with a quantum yield ranging from 0.007 (vitamin D<sub>3</sub>) to 0.64 (nicotinamide) independent of the excitation wavelength. Moreover, pre-irradiation of vitamins with UVB changed their absorption in the UVB and UVA spectral range. Subsequently, molecules such as vitamin E and vitamin K<sub>1</sub>, which normally exhibit no singlet oxygen generation in the UVA, now produce singlet oxygen when exposed to UVA at 355 nm. This interplay of different UV sources is inevitable when applying serial or parallel irradiation with UVA and UVB in experiments *in vitro*. These results should be of particular importance for parallel irradiation with UVA and UVB *in vivo*, e.g. when exposing the skin to solar radiation.

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## Introduction

Radiation of the ultraviolet spectral range is known to be hazardous to human health by inducing inflammation, cataract formation in the eye, premature skin aging and skin cancer.<sup>1,2</sup> In the US, skin cancer is the most frequent cancer showing an incidence of about 40 percent of all diagnosed human cancers.<sup>3</sup> The interaction of UV radiation with tissue depends on its wavelength. UV radiation is divided into UVC (<280 nm), UVB (280–320 nm) and UVA (320–400 nm) radiation. UVC radiation is almost completely absorbed in the atmosphere. UVB and UVA radiation is only partially absorbed in the atmosphere. Therefore solar radiation on the earth comprises UVA (~95%) and UVB (~5%).<sup>4</sup> The UVA spectrum is subdivided depending on the wavelength into UVA2 (320–340 nm) and UVA1 (340–400 nm), which is based on biological effects such as solar-UV-signature mutations in mouse skin<sup>5</sup> and on skin effects in phototherapy.<sup>6</sup> Up to 50% of UVA can reach the depth of melanocytes and the dermal compartment, whereas 14% of UVB reaches the lower epidermis.<sup>4</sup>

It is commonly accepted that UVB radiation is directly absorbed in cellular DNA, which typically leads to the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone photoproducts (6-4PP).<sup>7</sup> Since UVA is only poorly absorbed by DNA or proteins, other molecules in tissue may absorb that radiation. In case such a molecule can act as an endogenous photosensitizer, energy or charge transfer may occur from its triplet T<sub>1</sub> state to other adjacent molecules yielding reactive oxygen species (ROS), in particular singlet oxygen (<sup>1</sup>O<sub>2</sub>).<sup>8–10</sup> The deleterious biological effects of UVA radiation are mediated by these ROS; among them, <sup>1</sup>O<sub>2</sub> plays a major role.<sup>11–14</sup>

A number of endogenous UVA-photosensitizers have been identified in the past few decades. Among them, some vitamins of the A and B group, as well as medical drugs, play a major role.<sup>10</sup> Being regularly present in cells of tissue, which are frequently exposed to UV radiation, vitamins are potential photosensitizers and targets of UV radiation induced cell damage in the skin and eyes.

Vitamins are organic chemical compounds, which are essential for most organisms but cannot be produced in sufficient quantities by the organism,<sup>15,16</sup> and are taken up with the diet.<sup>16,17</sup> Humans need different aqueous and liposoluble vitamins because of their important role as cofactors or coenzymes in human metabolism reactions.<sup>17,18</sup> One of the

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most important benefits claimed for vitamins A, C, E and many of the carotenoids is their role as antioxidants, which are scavengers of free radicals, in particular when synergistic effects occur.<sup>19</sup> However, some vitamins such as B<sub>3</sub>, D<sub>2</sub>, D<sub>3</sub>, and E were not considered as endogenous UVA-photosensitizers because these molecules do not absorb UVA radiation.

From a photophysical point of view, the photosensitized generation of <sup>1</sup>O<sub>2</sub> should also be possible with UVB radiation, in particular because many known endogenous UVA-photosensitizers also absorb UVB radiation, even to a higher extent than UVA radiation (Fig. 1). Thus, UVB induced <sup>1</sup>O<sub>2</sub> might play an additional, important role in the mechanisms of oxidative tissue damage. However, this has rarely been investigated in the past few decades.<sup>20–22</sup> Vitamin E (α-tocopherol) was found to generate <sup>1</sup>O<sub>2</sub> under UVB-irradiation, and its functional efficiency as an antioxidant is now under discussion.<sup>20</sup> As a first step, we recently found that riboflavin, pyridoxine hydrochloride, and nicotinic acid produced <sup>1</sup>O<sub>2</sub> with a yield of 0.05 to 0.40 when exposed to 308 nm (UVB).<sup>23</sup>

In the present study, we firstly investigated the <sup>1</sup>O<sub>2</sub> generation of a series of vitamins<sup>17,24</sup> (Fig. 1) when exposed to UVB radiation. Secondly, the molecular structure may change when vitamins are exposed to UVB. This in turn may lead to a change of the absorption coefficient of such molecules in the entire range of ultraviolet radiation and hence change the ability to generate <sup>1</sup>O<sub>2</sub>.<sup>23</sup> Therefore, we pre-irradiated the

vitamins with UVB and determined subsequently the quantum yield of <sup>1</sup>O<sub>2</sub> generation for UVA radiation.

## Materials and methods

### Chemicals

5,10,15,20-Tetrakis(*N*-methyl-4-pyridyl)-21*H*,23*H*-porphine (TMPyP), sulfonated perinaphthene (PNS) and perinaphthene (PN) were used as reference photosensitizers to determine <sup>1</sup>O<sub>2</sub> quantum yield of the endogenous vitamins. TMPyP and PNS are well soluble in aqueous solutions like water (H<sub>2</sub>O) and deuterium oxide (D<sub>2</sub>O) with <sup>1</sup>O<sub>2</sub> quantum yields of 0.77 ± 0.04<sup>24</sup> or close to unity.<sup>25</sup> PN is well soluble in ethanol solution with a <sup>1</sup>O<sub>2</sub> quantum yield of 0.93 ± 0.08.<sup>26</sup> As aqueous soluble vitamins riboflavin, riboflavin 5′monophosphate sodium salt hydrate (FMN), flavin adenin dinucleotide (FAD), nicotinic acid, nicotinamide, pyridoxine (PYR), pyridoxine hydrochloride (PYR-HCL), pyridoxamine dihydrochloride (PYRINE-DHCL), pyridoxal hydrochloride (PYRXAL-HCL) and pyridoxal 5′phosphate hydrate (PYR-5-PH) were investigated. All these vitamins were dissolved preferably in H<sub>2</sub>O or D<sub>2</sub>O with different concentrations to achieve an appropriate absorption at the excitation wavelength between 30% and 80% using a cuvette with 1 cm thickness. The choice of aqueous solvent depended on the signal intensity of <sup>1</sup>O<sub>2</sub> luminescence. As lipophilic vitamins retinal all-*trans* (vitamin A), ergocalciferol (vitamin D<sub>2</sub>), cholecalciferol (vitamin D<sub>3</sub>), DL-α-tocopherol (vitamin E) and phylochinon (vitamin K<sub>1</sub>) were investigated. All these substances were solved in ethanol under the same conditions as the aqueous soluble substances. All substances were purchased from Sigma-Aldrich, Steinheim, Germany, except PN (Acros Organics, Geel, Belgium) and PNS, nicotinic acid and nicotinamide (Institute of Organic Chemistry, University of Regensburg, Germany).

### Absorption spectra

The transmission spectra of each solution probe were measured with a one-beam spectrophotometer (DU640, Beckman Instruments GmbH, Munich, Germany) using a quartz cuvette with an optical path of 1 cm (QS-101, Hellma Optik, Jena, Germany). The absorption values were calculated as  $A = 100\% - T$ , where  $T$  is the transmission value.

### Oxygen concentration

The oxygen concentration in solution was measured in a quartz cuvette with a needle sensor (MICROX TX, PreSens GmbH, Regensburg, Germany).

### Luminescence experiments

The solutions of the different molecules were transferred into a cuvette (QS-101, Hellma Optik, Jena, Germany) and excited by using a tunable laser system which is based on a Nd:YAG pump laser, with a pulse energy smaller than 3.15 mJ, a pulse duration of 7 ns and a repetition rate of 1 kHz. The laser emission is continuously tunable from 210 to 2600 nm, but for the

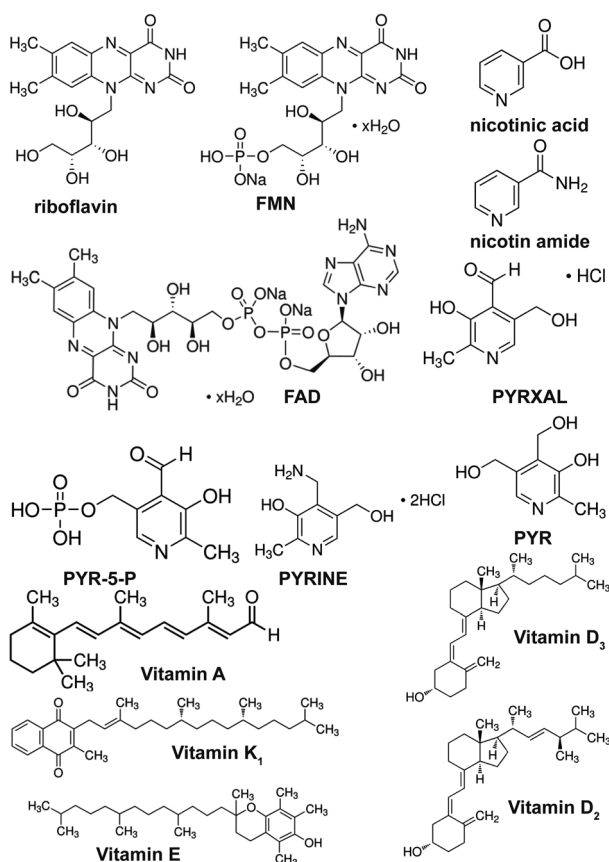


Fig. 1 The molecular structures of the investigated vitamins.



experiments excitation wavelengths in the UVB (308 nm) and UVA (330, 355, 370 nm) ranges were used. The laser intensity, which reaches the cuvette, depends on the wavelength and ranged in the experiments between 15 and 50 mW cm<sup>-2</sup>. The <sup>1</sup>O<sub>2</sub> luminescence (1270 nm) was detected using a photomultiplier (R5509-42, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany).<sup>27</sup> For spectral resolution, luminescence was detected at wavelengths from 1150 to 1350 nm by using interference filters in front of the photomultiplier.

### Spectrally resolved <sup>1</sup>O<sub>2</sub> luminescence

The total sum of all detected luminescence photons is calculated at the detected wavelengths given by the respective interference filters. A curve was fitted (Lorentz-shape) to the sum of the single values. The values are normalized to the maximum of the curve.

### Determination of <sup>1</sup>O<sub>2</sub> luminescence decay and rise time

The decay and rise time of the <sup>1</sup>O<sub>2</sub> luminescence signal are given by the following formula:<sup>28,29</sup>

$$I(t) = \frac{C}{\tau_R^{-1} - \tau_D^{-1}} \left[ \exp\left(-\frac{t}{\tau_D}\right) - \exp\left(-\frac{t}{\tau_R}\right) \right] \quad (1)$$

The constant *C* was used to fit the luminescence signal (Mathematica 8.0, Wolfram Research, Berlin, Germany) yielding the decay and rise times (*t<sub>D</sub>*, *t<sub>R</sub>*). The experimental accuracy was estimated to be between 10 and 20% of the value determined by the fit.

### Determination of the <sup>1</sup>O<sub>2</sub> quantum yield

To estimate the quantum yield of the analyzed endogenous molecules, the measured luminescence signals were compared to the signals of the respective reference photosensitizer with known quantum yield  $\Phi_{\Delta}$ . The quantum yields of aqueous soluble molecules were compared to the quantum yields of PNS and TMPyP and the quantum yield of ethanol soluble molecules to PN and TMPyP. The endogenous molecule and the reference photosensitizer were dissolved in the corresponding solvent in different concentrations. Because the oxygen concentration has an influence on the quantum yields for all measurements, air saturated solutions were used. First the absorption of the different solutions at the excitation wavelength (EW) was calculated to determine the absorbed energy *E<sub>abs</sub>* of each substance with the laser power at the EW. Then the <sup>1</sup>O<sub>2</sub> luminescence signals were measured for different concentrations of the endogenous molecule and the reference substance at the EW. The sum of all <sup>1</sup>O<sub>2</sub> luminescence photons was used to estimate the luminescence energy *E<sub>lum</sub>*.

Using reference photosensitizers, the quantum yield  $\Phi_{\Delta}^2$  was calculated by comparing the slopes *S* of the luminescence energy *E<sub>lum</sub>* of the <sup>1</sup>O<sub>2</sub> luminescence (unknown or reference) versus the absorbed laser energy *E<sub>abs</sub>* with the following formula:<sup>28</sup>

$$\frac{S^2([O_2])}{S^{ref}([O_2])} = \frac{\Phi_{\Delta}^2([O_2])}{\Phi_{\Delta}^{ref}([O_2])} \quad (2)$$

### Photostability

For investigating the photostability of the vitamins in the UVB range, a broadband UVB Transluminator (FLX-20M Transluminator, Biometra GmbH, Göttingen, Germany) was used. The UVB tubes of the Transluminator have an emission maximum at 312 nm and the cuvettes could be irradiated equally from one side with a specific UVB radiant exposure (J cm<sup>-2</sup>).

## Results and discussion

Vitamins are essential compounds for many functions of the human organism including the skin. Some vitamins can be synthesized, but others need to be obtained by an adequate diet. In addition, vitamins are applied topically for skin care and to treat skin diseases. The human epidermis contains significant amounts of vitamin A (all-*trans*-retinol), enzymes responsible for its metabolism, binding proteins for its protection and transport, and the nuclear receptors involved in the respective induced gene activity modulation. Vitamin A normalizes keratinization and can be topically applied to treat acne or photodamage of the skin.<sup>30–32</sup> Vitamin E, like vitamin A, is present in mammalian skin. The probable physiological function of epidermal vitamin E is to contribute to the antioxidant defense of the skin, usually in combination with vitamin C.

Vitamin B<sub>3</sub>, or nicotinamide or niacinamide, is a derivative of niacin obtained through diet from meat, fish, milk, eggs, and nuts. Nicotinamide is part of the coenzymes nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), and its reduced forms are NADH and NADPH. Clinical studies have presented results on its anti-inflammatory actions. Nicotinamide is responsible for the synthesis of sphingolipids, free fatty acids, cholesterol, and ceramides, thus decreasing trans-epidermal water loss.

Vitamin C is a very important antioxidant in human skin. However, even extensive oral supplementation leads to a limited increase of vitamin C in skin only. Therefore, topical application of vitamin C is an appropriate way to increase its concentration in the skin. Vitamin C supports the formation of stratum corneum barrier lipids and regenerates vitamin E. Vitamin K displays antihemorrhagic properties and topical application of vitamin K has been used for the prevention of vascular manifestations of aging, for the suppression of pigmentation and for the resolution of bruising.

Solar radiation is the major source of UV radiation that interacts with cells of the skin and eye and can be absorbed by vitamins present in such cells. For example, UV is important for the synthesis of vitamin D<sub>3</sub> but at the same time, vitamins and UV can affect the integrity of the skin and eye lens via photosensitized production of <sup>1</sup>O<sub>2</sub>. This reactive oxygen species can yield products like 8-oxo-7,8-dihydro-2'-deoxyguanosine (8oxodGuo).<sup>4,11,33,34</sup> This conflicting potential of vitamins was already known for some substances in correlation with UVA radiation. Many vitamins show high absorption values in the UVB range 280–320 nm compared to the UVA



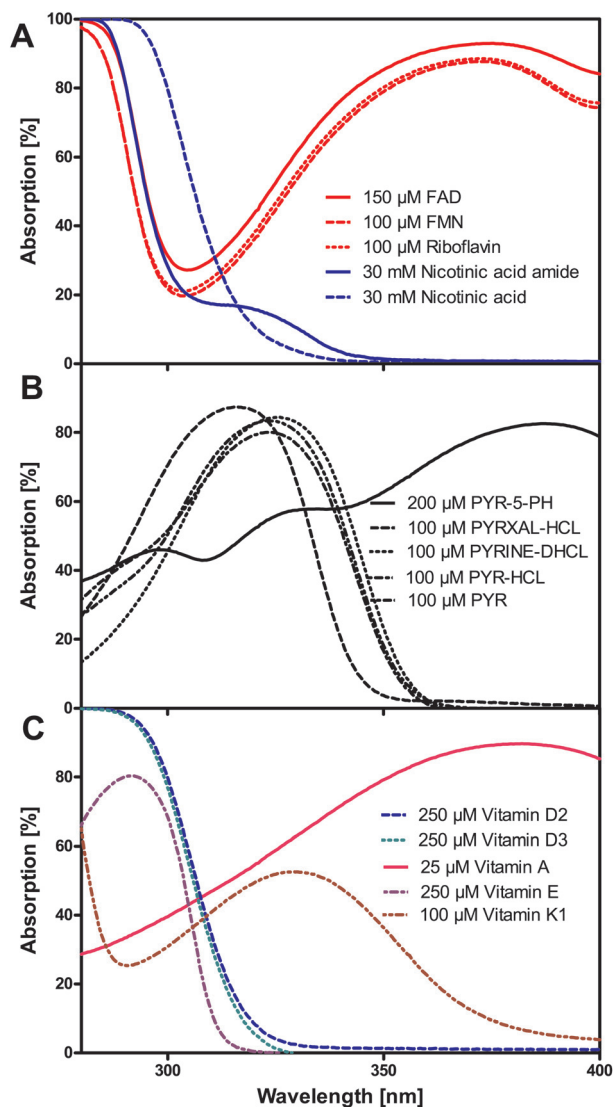


Fig. 2 The measured absorption spectra of the different investigated vitamins are shown in the UVB and UVA range (280–400 nm). The vitamins were dissolved in aqueous solution (A, B) or ethanol (C) and the absorption spectra were measured without any further irradiation.

(320–400 nm) range. Fig. 2 shows the absorption spectra of investigated vitamins from 280–400 nm. The substances were dissolved with an appropriate concentration to provide absorption values between 20 and 80% when excited in the UVB (308 nm). The high UVB absorption of vitamins is an excellent prerequisite for photosensitized production of  $^1\text{O}_2$  that was subsequently measured.

#### $^1\text{O}_2$ luminescence detection of vitamins excited at 308 nm

The aqueous soluble vitamins FAD (150  $\mu\text{M}$ ), FMN (200  $\mu\text{M}$ ), riboflavin (200  $\mu\text{M}$ ), PYR-5-PH (100  $\mu\text{M}$ ), PYRXAL-HCL (100  $\mu\text{M}$ ), PYRINE-DHCL (100  $\mu\text{M}$ ), PYR-HCL (100  $\mu\text{M}$ ), PYR (100  $\mu\text{M}$ ), nicotinic acid (30 mM) and nicotinamide (30 mM) were dissolved in  $\text{D}_2\text{O}$  and excited at 308 nm. The lipophilic vitamin A (30  $\mu\text{M}$ ), vitamin  $\text{D}_2$  (250  $\mu\text{M}$ ), vitamin  $\text{D}_3$  (250  $\mu\text{M}$ ),

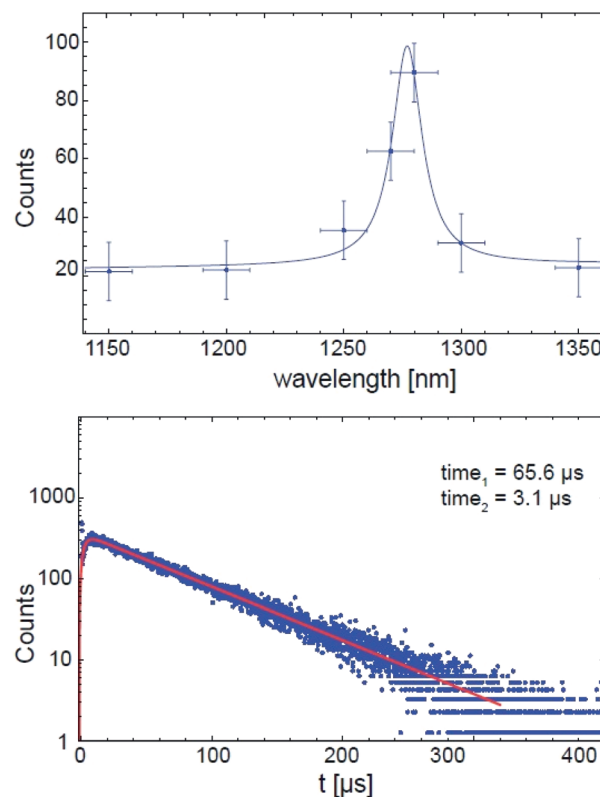


Fig. 3 The time-resolved  $^1\text{O}_2$  signal of 200  $\mu\text{M}$  FMN in  $\text{D}_2\text{O}$  is shown together with the fitted decay and rise times (bottom). On the top the respective spectral resolution for FMN is shown with a clear peak between 1270 and 1280 nm.

vitamin E (250  $\mu\text{M}$ ) and vitamin  $\text{K}_1$  (100  $\mu\text{M}$ ) were dissolved in ethanol and also excited at 308 nm.

All substances revealed clear time- and spectrally resolved  $^1\text{O}_2$  luminescence signals, exemplarily shown for FMN (Fig. 3). The substances FMN, riboflavin, nicotinic acid and nicotinamide showed luminescence decay times in the range of 65 to 67  $\mu\text{s}$ , which corresponds well with the  $^1\text{O}_2$  decay time in  $\text{D}_2\text{O}$  (66  $\mu\text{s}$ ).<sup>35</sup> PYR-5-PH, PYRXAL-HCL, PYRINE-DHCL, PYR-HCL and PYR solutions yielded luminescence decay times in the range of 46 to 54  $\mu\text{s}$ , which is clearly shorter than the  $^1\text{O}_2$  decay time in  $\text{D}_2\text{O}$ , but in agreement with the results of Dzhangarov *et al.* for vitamin  $\text{B}_6$ .<sup>36</sup> This shortening of  $^1\text{O}_2$  decay time is mainly caused by quenching of  $^1\text{O}_2$  decay by the photosensitizer molecule itself (self-quenching).<sup>37</sup> PYR-5-PH, PYRXAL-HCL, PYRINE-DHCL, PYR-HCL and PYR are all known  $^1\text{O}_2$  quenchers.<sup>38</sup> This effect was more pronounced for FAD, which showed a decay time of about 28  $\mu\text{s}$ .

Concerning the lipophilic vitamins, the fitted decay of vitamin A in ethanol was 14  $\mu\text{s}$ , which corresponds well to the  $^1\text{O}_2$  decay time in this solvent.<sup>39</sup> For vitamin E the fitted decay time was about 9  $\mu\text{s}$ , which is considerably shorter than the  $^1\text{O}_2$  decay time in ethanol. This is probably due to the fact that vitamin E is a good quencher of  $^1\text{O}_2$ .<sup>40</sup> The luminescence signals of vitamin  $\text{D}_2$ ,  $\text{D}_3$  and  $\text{K}_1$  were too weak to fit rise and decay times (eqn (1)).





The oxygen concentration in solution decreased for some vitamins during irradiation (308 nm, 35 mW). After 20 minutes, the maximum decrease was found in solutions of vitamin E (88.5%) and vitamin K<sub>1</sub> (40.2%). The percentage values represent the decrease with reference to the initial value before irradiation. The B<sub>6</sub> vitamins and vitamin A showed a decrease of oxygen concentration in the range of 7.0 to 35.0%. Nicotinamide and vitamin D<sub>2</sub> showed a decrease of 2.0% and 10.0%, respectively, whereas nicotinic acid and vitamin D<sub>3</sub> showed no decrease within experimental accuracy. This supports the assumption that these molecules may react with <sup>1</sup>O<sub>2</sub> being a generator and a quencher of <sup>1</sup>O<sub>2</sub> at the same time.

The spectrally resolved luminescence signals of the aqueous soluble vitamins showed a maximum in the range of 1269 to 1277 nm, which is in line with the transition energy of the excited (<sup>1</sup>O<sub>2</sub>) to the ground state (<sup>3</sup>O<sub>2</sub>) of oxygen in aqueous solvents.<sup>37</sup> The spectrally resolved <sup>1</sup>O<sub>2</sub> luminescence signal of the lipophilic vitamins had a maximum at 1274 ± 2 nm, which is likewise in line with the transition energy of oxygen in ethanol.<sup>39</sup> Since all experiments show this spectrally resolved maximum of luminescence, the detected signals clearly provide evidence of <sup>1</sup>O<sub>2</sub> production of these vitamins when excited with UVB.

### Quantum yield of <sup>1</sup>O<sub>2</sub> generation

The efficacy of <sup>1</sup>O<sub>2</sub> generation of a specific photosensitizer ( $\Phi_{\Delta}$ ) critically depends on the intersystem crossing quantum yield of the photoexcited molecule and on the efficiency of energy transfer to oxygen. The values of  $\Phi_{\Delta}$  were measured for all vitamins in solution at 308 nm (UVB). The well-known photosensitizers PNS and TMPyP were used as reference for aqueous soluble vitamins and PN and TMPyP served as reference for lipophilic vitamins. All aqueous soluble vitamins were dissolved in H<sub>2</sub>O at different concentrations, except nicotinic

acid and nicotinamide, which were dissolved in D<sub>2</sub>O to achieve sufficiently high luminescence signals. The lipophilic vitamins were dissolved in ethanol at different concentrations. The <sup>1</sup>O<sub>2</sub> luminescence decay time of all vitamins and reference photosensitizers was either 3.5 ± 0.4 μs (H<sub>2</sub>O) or in the range of 65 to 67 μs (D<sub>2</sub>O).

### Quantum yield of <sup>1</sup>O<sub>2</sub> generation using UVB excitation

We recently investigated a small number of bio-molecules regarding <sup>1</sup>O<sub>2</sub> generation under UVB radiation.<sup>23</sup> Besides some fatty acids, riboflavin, PYR-HCL, and nicotinic acid generated <sup>1</sup>O<sub>2</sub> when exposed to 308 nm. Now, we could extend the list of endogenous UVB photosensitizers and show that all investigated vitamins exhibit a quantum yield for <sup>1</sup>O<sub>2</sub> generation when exposed to UVB radiation at 308 nm. As displayed in Table 1, the values ranged from 0.007 ± 0.005 for vitamin D<sub>3</sub> to 0.64 ± 0.07 for nicotinamide. Among the investigated vitamins, only vitamin E is already known to generate <sup>1</sup>O<sub>2</sub> via excitation at 308 nm.<sup>20</sup> The reported quantum yield was 0.17 ± 0.025 in methanol, which is in good correlation with our experiments (0.15 ± 0.02, Table 1).

Our values of quantum yields differ for PYR, PYR-5-PH, PYRXAL-HCL, and PYR-HCL, when compared to Dzshagarov *et al.* (Table 1).<sup>41</sup> However, we learnt from our setting used previously<sup>23</sup> that the power stability of the exciting UVB source during <sup>1</sup>O<sub>2</sub> detection is extremely critical for the accuracy of <sup>1</sup>O<sub>2</sub> quantum yield. Our laser system offers an excellent stability of optical power of less than 3.5% fluctuation during <sup>1</sup>O<sub>2</sub> detection.

### Quantum yield of <sup>1</sup>O<sub>2</sub> generation using different UVA wavelengths

UVA1 phototherapy (wavelength: 340–400 nm) has become a new physical therapy in recent years, which is effective in the

**Table 1** The <sup>1</sup>O<sub>2</sub> quantum yield  $\Phi_{\Delta}$  of endogenous photosensitizers at different UV wavelengths

| Endogenous photosensitizers       | UVB<br>308 nm | UVA2<br>330 nm | UVA1<br>370 nm | Literature values             |
|-----------------------------------|---------------|----------------|----------------|-------------------------------|
| FAD                               | 0.13 ± 0.03   | 0.12 ± 0.03    | 0.15 ± 0.03    | 0.07 ± 0.01 <sup>d 28</sup>   |
| FMN                               | 0.58 ± 0.06   | 0.64 ± 0.07    | 0.58 ± 0.06    | 0.51 ± 0.07 <sup>d 28</sup>   |
| Riboflavin                        | 0.61 ± 0.07   | 0.64 ± 0.07    | 0.58 ± 0.06    | 0.54 ± 0.07 <sup>d 28</sup>   |
|                                   |               |                |                | 0.40 ± 0.10 <sup>e 23</sup>   |
| PYR-5-PH                          | 0.16 ± 0.03   | 0.13 ± 0.03    | 0.14 ± 0.03    | 0.54 ± 0.06 <sup>c 41</sup>   |
| PYRXAL-HCL                        | 0.06 ± 0.02   | 0.08 ± 0.02    | — <sup>a</sup> | 0.44 ± 0.06 <sup>c 36</sup>   |
| PYRINE-DHCL                       | 0.06 ± 0.02   | 0.04 ± 0.01    | — <sup>a</sup> | 0.06 ± 0.02 <sup>c 41</sup>   |
| PYR-HCL                           | 0.07 ± 0.02   | 0.05 ± 0.02    | — <sup>a</sup> | 0.28 ± 0.04 <sup>c 41</sup>   |
|                                   |               |                |                | 0.10 ± 0.05 <sup>e 23</sup>   |
| PYR                               | 0.11 ± 0.03   | 0.08 ± 0.02    | — <sup>a</sup> | 0.28 ± 0.04 <sup>c 41</sup>   |
| Nicotinic acid <sup>f</sup>       | 0.05 ± 0.01   | — <sup>a</sup> | — <sup>a</sup> | 0.05 ± 0.05 <sup>e 23</sup>   |
| Nicotinic acid amide <sup>f</sup> | 0.64 ± 0.07   | — <sup>a</sup> | — <sup>a</sup> | — <sup>b</sup>                |
| Vitamin A                         | 0.06 ± 0.02   | —              | —              | 0.05 <sup>d 55</sup>          |
| Vitamin D <sub>2</sub>            | 0.02 ± 0.01   | — <sup>a</sup> | — <sup>a</sup> | — <sup>b</sup>                |
| Vitamin D <sub>3</sub>            | 0.007 ± 0.005 | — <sup>a</sup> | — <sup>a</sup> | — <sup>b</sup>                |
| Vitamin E                         | 0.15 ± 0.02   | — <sup>a</sup> | — <sup>a</sup> | 0.085 ± 0.005 <sup>e 20</sup> |
|                                   |               |                |                | 0.17 ± 0.025 <sup>e 20</sup>  |
| Vitamin K <sub>1</sub>            | 0.02 ± 0.01   | —              | —              | — <sup>b</sup>                |

<sup>a</sup> Absorption of radiation at the respective wavelength was too low. <sup>b</sup> No literature values available. <sup>c</sup> Vitamins of the B<sub>6</sub> group without residual.

<sup>d</sup> Excitation at 355 nm. <sup>e</sup> UVB excitation. <sup>f</sup> Dissolved in D<sub>2</sub>O.



treatment of scleroderma and other connective tissue diseases.<sup>42</sup> UVA1 damage to critical biomolecules such as DNA has been widely attributed to its ability to generate reactive oxygen species *via* endogenous photosensitizers.<sup>43</sup> However, UVA radiation was as effective as solar simulated UV (290–400 nm) in suppressing the elicitation of an established immune response, whereas irradiation with UVA1 had no effect.<sup>44</sup> Other experiments showed that a specific spectrum, which contained UVB and UVA2 (290–325 nm), augments the formation of melanocytic nevi in a guinea-pig model for human nevi.<sup>45</sup> It was proved that the solar-UV-signature mutation in mouse skin is commonly observed in the wavelength range from UVB to UVA, and that UVA1 induced the mutation more preferentially than the other shorter wavelength ranges.<sup>5</sup> It was recently shown, both *in vivo* and *in vitro*, that UVA1 radiation suppresses calcineurin activity and that this activity loss is due to singlet oxygen and superoxide generated by photosensitized reactions. <sup>1</sup>O<sub>2</sub> obviously plays a certain role in these UVA1 or UVA2 mediated processes and the vitamins are potential endogenous photosensitizers.

However, these different findings might lead to the suggestion that different UVA wavelengths might yield different quantum yields of <sup>1</sup>O<sub>2</sub> generation. The values of  $\Phi_{\Delta}$  were already determined for some vitamins when using either broadband UVA radiation (320–400 nm) or monochromatic UVA laser excitation with usually 355 nm.<sup>10</sup> Now, our endogenous photosensitizers were exposed to wavelengths of either UVA1 (320–340 nm) or UVA2 (340–400 nm) to check differences of <sup>1</sup>O<sub>2</sub> generation. We measured the values of  $\Phi_{\Delta}$  in the UVA1 and UVA2 range by exciting the vitamins in solution at 330 nm (UVA2) or 370 nm (UVA1) provided that the respective vitamin molecule showed sufficient absorption at these wavelengths. The quantum yield  $\Phi_{\Delta}$  could be detected for FAD, FMN, riboflavin and PYR-5-PH in both UVA ranges (Table 1). Due to the absorption coefficient of PYRXAL-HCL, PYRINE-DHCL, PYR-HCL and PYR, the values could be detected for the excitation with UVA2 only (Table 1). The vitamins of the B-group contribute to UVA induced <sup>1</sup>O<sub>2</sub> generation, whereas the effect is restricted for some of them to UVA2 radiation due to their absorption spectra.

When taking the values of  $\Phi_{\Delta}$  for UVA1, UVA2, and even UVB into account, the choice of excitation wavelength seems to play no role within experimental accuracy. Whether these vitamins generate <sup>1</sup>O<sub>2</sub> depends obviously on their ability to absorb the respective radiation. The values of  $\Phi_{\Delta}$  are in line with the values found in the literature for FMN, FAD, riboflavin, vitamins A and E. However, our values for the vitamin B<sub>6</sub> molecules are constantly smaller ( $\leq 0.16$ ) as compared to the work of Dzhangarov *et al.*, except for PYRINE-DHCL. One other possible explanation could be the method of estimating the  $\Phi_{\Delta}$  value by comparing with a reference substance. Dzhangarov *et al.* used the maximal amplitude of the luminescence signal as luminescence energy. In our experiments, all detected luminescence photons of the luminescence signal were summed up to calculate  $\Phi_{\Delta}$ . This procedure considers also the

shortened luminescence decay times of B<sub>6</sub> vitamins and might avoid an overestimation of  $\Phi_{\Delta}$  values.

### Photostability

Absorption of radiation by an endogenous photosensitizer such as vitamins and its ability to produce <sup>1</sup>O<sub>2</sub> are very sensitive to the respective molecular structure. Any change of that structure can immediately and substantially change absorption and <sup>1</sup>O<sub>2</sub> production. Energy-rich UV radiation can change the structure of photosensitizer molecules depending on the wavelength and the energy applied. These changes, in particular during continuous, long-lasting irradiation, should affect the role of endogenous photosensitizers and its impact on cellular damage. It is already known that UV radiation can lead to photochemical decomposition of vitamin molecules such as  $\alpha$  and  $\gamma$  tocopherol,<sup>46,47</sup> vitamin A,<sup>48,49</sup> vitamin K<sub>1</sub>,<sup>50</sup> and vitamin B.<sup>51,52</sup>

Therefore, the absorption spectra of the vitamins were recorded after UVB irradiation at 1 J cm<sup>-2</sup> using a UVB lamp showing an emission maximum at 312 nm (Fig. 4, red line). This radiant exposure equals roughly the daily radiant exposure of UVB in Potsdam, Germany.<sup>53</sup> The mean UVB intensity used for estimating <sup>1</sup>O<sub>2</sub> quantum yields at 308 nm is 0.45  $\pm$  0.05 J cm<sup>-2</sup>. Absorption spectroscopy showed that the UVB irradiation of the vitamins changed the absorption spectrum in different ways, partially in the entire UV range (280–400 nm). For example, UVB and UVA absorption of PYR-HCL decreased, but increased for vitamin K<sub>1</sub> (Fig. 4). In the case of PYR-5-PH, the absorption in the UVB region increased, but decreased in the UVA range, and the other way around for vitamin E (Fig. 4).

For all vitamins the absorption spectra before (Fig. 4, blue line) and after (Fig. 4, red line) irradiation with 1 J cm<sup>-2</sup> were measured and analyzed. The difference in absorption at 308 nm in the UVB and at 355 nm in the UVA region before and after irradiation is shown for all vitamins (Table 2). Also the integral of the whole absorption in the UVB (280–320 nm) and in the UVA (320–400 nm) region was calculated before and after the irradiation and compared with each other (Table 2). For nearly all vitamins a clear difference in absorption at least in the UVA or UVB region after UVB irradiation could be detected. Only the absorption spectra of FMN showed no difference after the UVB irradiation (1 J cm<sup>-2</sup>) within the experimental accuracy.

### <sup>1</sup>O<sub>2</sub> generation of UVB pre-irradiated vitamins

These altered molecules might show different photophysical features. Exemplarily, the <sup>1</sup>O<sub>2</sub> generation of the lipophilic vitamin E and vitamin K<sub>1</sub> before and after UVB pre-irradiation was investigated for an excitation wavelength of 308 and 355 nm. Vitamin E shows UVA absorption close to zero before UVB pre-irradiation at 355 nm. Vitamin K<sub>1</sub> shows high absorption in the UVA range but a very low singlet oxygen quantum yield (0.02  $\pm$  0.01). Therefore, vitamin E and vitamin K<sub>1</sub> without pre-irradiation showed a very low <sup>1</sup>O<sub>2</sub> luminescence signal when excited with UVA radiation.



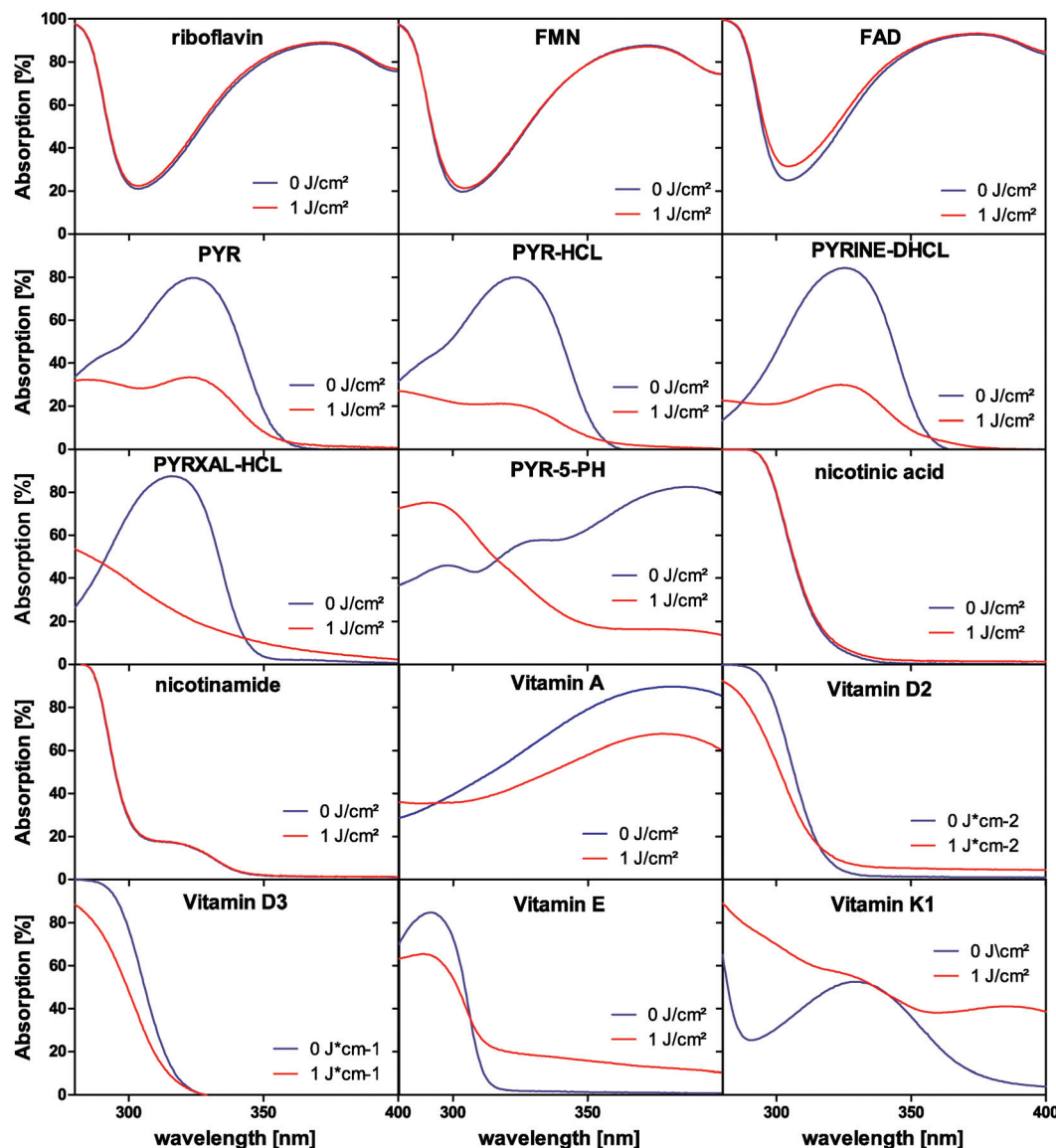


Fig. 4 The changes of the absorption spectra of all investigated vitamins after irradiation with  $1 \text{ J cm}^{-2}$  UVB (red line) are shown. The blue line represents the original absorption spectra of each molecule.

However, when vitamin E was exposed to  $1 \text{ J cm}^{-2}$  UVB radiation, the absorption value at  $355 \text{ nm}$  increased from 1 to 15%. After that, vitamin E was excited with  $355 \text{ nm}$  laser radiation and the spectrally resolved luminescence signal clearly showed  $^1\text{O}_2$  generation (Fig. 5) with  $\Phi_{\Delta} = 0.05 \pm 0.01$ . However, this value is smaller than for excitation with UVB at  $308 \text{ nm}$  ( $0.15 \pm 0.02$ , Table 1).

The absorption of vitamin  $\text{K}_1$  at  $355 \text{ nm}$  increased for irradiation with  $1 \text{ J cm}^{-2}$  UVB radiation from 28% to 38% (Table 2). Comparable to experiments with vitamin E, pre-irradiation of vitamin  $\text{K}_1$  with UVB led to  $^1\text{O}_2$  generation when excited with  $355 \text{ nm}$  (Fig. 4). The corresponding value of  $\Phi_{\Delta}$  increased from  $0.02 \pm 0.01$  (Table 1) to  $0.10 \pm 0.02$ .

The absorption of vitamin E and  $\text{K}_1$  also increased at  $308 \text{ nm}$  after irradiation with  $1 \text{ J cm}^{-2}$  UVB radiation (Table 2).

The spectrally resolved singlet oxygen luminescence signal of vitamin  $\text{K}_1$  *via* UVB excitation ( $308 \text{ nm}$ ) also increased after pre-irradiation with  $1 \text{ J cm}^{-2}$  UVB radiation (Fig. 5). The corresponding value of  $\Phi_{\Delta}$  increased from  $0.02 \pm 0.01$  (Table 1) to  $0.10 \pm 0.02$  just as for excitation at  $355 \text{ nm}$ . For vitamin E the spectrally resolved singlet oxygen signal decreased for an excitation at  $308 \text{ nm}$  after UVB pre-irradiation, which is in line with a decreased singlet oxygen quantum yield  $\Phi_{\Delta}$  of  $0.05 \pm 0.01$ .

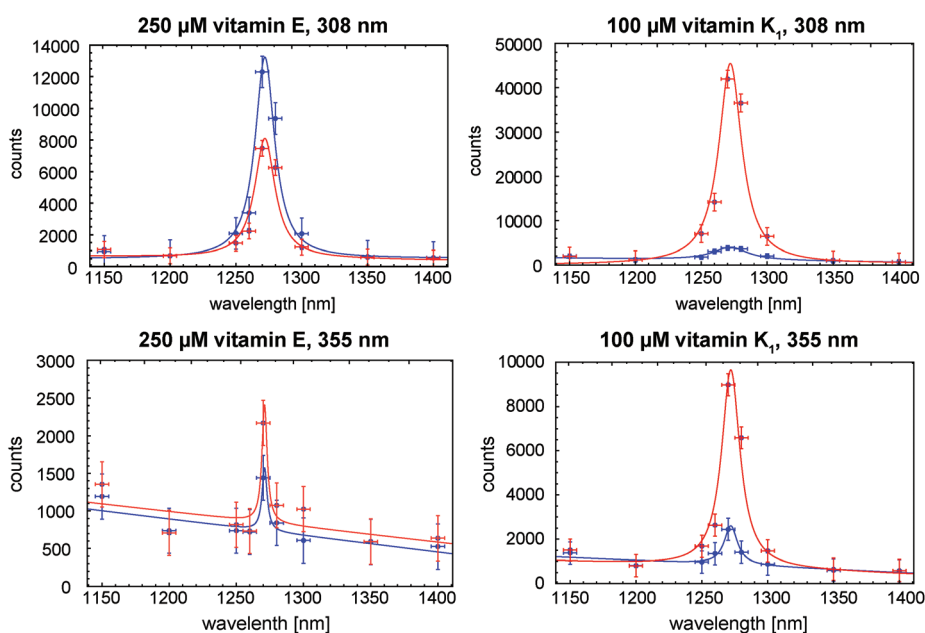
These data provide evidence that irradiation of vitamins with UVB can change the photophysical features of these endogenous photosensitizers, leading to a change of their potential to generate  $^1\text{O}_2$  when exposed to UVB or UVA radiation. Thus, UVB photosensitizers like vitamin E also become endogenous photosensitizers for UVA radiation after exposure to UVB.



**Table 2** The changes of the absorption of endogenous vitamins before and after irradiation with  $1 \text{ J cm}^{-2}$  UVB<sup>a</sup>

| Substance                                   | UVB (308 nm)<br>absorption [%] |       | UVA (355 nm)<br>absorption [%] |       | Total UVB absorption<br>(280–320 nm) | Total UVA absorption<br>(320–400 nm) |
|---|--------------------------------|-------|--------------------------------|-------|--------------------------------------|--------------------------------------|
|   | Before                         | After | Before                         | After | After                                | After                                |
| FAD (150 $\mu\text{M}$ )                    | 26                             | 32    | 88                             | 89    | +8%                                  | +2%                                  |
| FMN (100 $\mu\text{M}$ )                    | 21                             | 21    | 83                             | 82    | Unchanged                            | Unchanged                            |
| Riboflavin (100 $\mu\text{M}$ )             | 23                             | 24    | 84                             | 85    | +3%                                  | +2%                                  |
| Nicotinic acid amide (30 mM)                | 18                             | 18    | 1                              | 1     | Unchanged                            | +4%                                  |
| Nicotinic acid (30 mM)                      | 42                             | 44    | 0                              | 2     | +1%                                  | +93%                                 |
| PYR-5-PH (200 $\mu\text{M}$ )               | 43                             | 61    | 67                             | 17    | +50%                                 | –69%                                 |
| PYRXAL-HCL (100 $\mu\text{M}$ )             | 83                             | 32    | 2                              | 9     | –41%                                 | –47%                                 |
| PYRINE-DHCL (100 $\mu\text{M}$ )            | 65                             | 24    | 9                              | 6     | –51%                                 | –62%                                 |
| PYR-HCL (100 $\mu\text{M}$ )                | 65                             | 21    | 6                              | 4     | –58%                                 | –70%                                 |
| PYR (100 $\mu\text{M}$ )                    | 66                             | 19    | 8                              | 5     | –60%                                 | –70%                                 |
| Vitamin A (25 $\mu\text{M}$ )               | 45                             | 37    | 82                             | 61    | –8%                                  | –26%                                 |
| Vitamin D <sub>2</sub> (250 $\mu\text{M}$ ) | 44                             | 31    | 1                              | 5     | –20%                                 | +448%                                |
| Vitamin D <sub>3</sub> (250 $\mu\text{M}$ ) | 40                             | 23    | 0                              | 0     | –28%                                 | –100%                                |
| Vitamin E (250 $\mu\text{M}$ )              | 25                             | 31    | 1                              | 15    | –11%                                 | +1047%                               |
| Vitamin K <sub>1</sub>                      | 38                             | 63    | 29                             | 38    | +93%                                 | +66%                                 |

<sup>a</sup> An increase or decrease in absorption ( $\geq 2\%$  for absorption at 308 or 355 nm) is indicated with bold numbers. The total absorption was calculated by summing up all absorption values in the spectral range (280–320 and 320–400 nm) before and after irradiation. The change in absorption after UVB pre-irradiation is given in %.



**Fig. 5** On the left side, the spectral-resolved  $^1\text{O}_2$  signal of 250  $\mu\text{M}$  vitamin E in ethanol is shown for UVB (308 nm, top) or UVA excitation (355 nm, bottom) before (blue line) and after (red line) pre-irradiation with a UVB radiant exposure of  $1 \text{ J cm}^{-2}$ . The maximum is fitted between 1270 and 1275 nm. The charts on the right side show the corresponding results for spectral-resolved  $^1\text{O}_2$  signal of 100  $\mu\text{M}$  vitamin K<sub>1</sub> in ethanol.

This effect should play a major role in all experimental settings in which UVA and UVB were consecutively applied to cells or tissue to detect any signaling or damaging effects.<sup>54</sup> These changes may even occur under parallel application of UVA and UVB radiation as for experiments with solar simulators or exposure to natural solar radiation. In addition, such photochemical changes of endogenous photosensitizers along with the ability to generate  $^1\text{O}_2$  may affect the interpretation of

results regarding the role of UVA and UVB in skin damaging effects *in vivo*.

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