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Quantitative Analysis of β-Sitosterol Oxides Induced in Vegetable Oils by Natural Sunlight, Artificially Generated Light, and Irradiation

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UV radiation is able to induce lipid peroxidation. Photooxidation-induced β-sitosterol oxides were monitored in four vegetable oils exposed to sunlight for 10, 20, and 30 days during May 2005 (northeastern France), exposed to artificial light generated by a high-pressure Hg lamp for 21, 42, and 63 h at room temperature, and exposed to a 10 MeV electron beam at 0.93, 2.69, and 9.30 kGy at 8 °C. Quantification was performed by capillary gas chromatography−mass spectrometry according to the total ion current mode and using a reconstructed ion trace chromatogram with specific ion fragments. Sunlight induced the formation of higher amounts of oxides than UV light, while no significant oxidizing effect was observed with electron beam irradiation. However, data suggested that the amount of the main oxides formed was strongly dependent on the dose rate (length of exposure). Accordingly, shorter but more intense treatments had lower oxidizing effects.

KEYWORDS: **β-Sitosterol oxides; oil; GC-MS; sunlight; UV; irradiation**

INTRODUCTION

The health-promoting effects of phytosterols (plant sterols), in particular their cholesterol-lowering effect (*1*, *2*), have been known for more than half a century. Reports have also shown that phytosterols may display anticancer activity (*3*, *4*). During the past decade, the interest over phytosterols has increased as functional foods enriched with these products are being launched commercially. This in turn triggered concerns about possible oxidation of phytosterols in these foods, the extent of such an oxidation, and the daily intake of phytosterol oxidation products. In fact, the chemical nature of phytosterols makes these molecules susceptible to oxidation and many factors such as heat and light may increase the oxidation rate. Information is however still scarce despite the strong demand for more efficient and accurate analytical techniques, which are crucial for the determination of the levels of phytosterol oxidation products in foods.

The knowledge in this area is somewhat limited to thermo-oxidation of cholesterol, although other oxidation catalyzers and some phytosterols have been studied. It was shown that UV radiation, heating (*5*−*7*), chemical catalysis (*8*), and enzymatic processes (*9*) are able to induce unsaturated lipid oxidation and peroxidation (*10*−*12*). With regard to photooxidation, cholesterol oxidation products were generated in spray-fried egg yolk (*13*), butter (*14*, *15*), model system liposomes (*16*, *17*), and human skin stratum corneum (*18*−*20*). In particular, studies by Chatterjee et al. (*16*) and Trommer et al. (*20*) showed that cholesterol and some other lipids may be oxidized by artificial UV light via singlet oxygen attack followed by a succession of free radical chain reactions.

Another source of concern with regard to sterol oxidation is food irradiation. This technology is widely reported as a safe and effective way to eliminate foodborne pathogens (*21*−*23*). However, some of its drawbacks include the destruction of vitamins (*24*, *25*), the induction of lipid oxides (*26*, *27*) and subsequent off-flavors, and the possible toxicity of radiolytic products (*28*). Studies about radioinduced oxidation, however, focused on meat products and therefore dealt mainly with oxidation products of cholesterol and other meat lipids.

As far as phytosterols are concerned, oxidation products from the major phytosterols, sitosterol, campesterol, and stigmasterol, could be generated through autoxidation at elevated temperatures (*29*, *30*). The oxidation mechanisms and products formed have been studied, and some analytical methods have been reported.

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However, much information in the area still remains to be discovered, in particular with regard to radio- and photoinduced phytosterol oxidation products. Because of a chemical structure close to that of cholesterol, in particular the presence of a double bond in the unsaturated B-ring of the cyclic nucleus, phytosterols can be expected to be as reactive to oxidation by light and irradiation.

In a previous investigation (*33*), we studied edible vegetable oils, namely, sunflower, soybean, olive, and rapeseed oils, and showed very significant contents of β-sitosterol. In the present study, oxidation products from this major phytosterol were generated in the various oils, commercially available and currently used in French cooking, through exposure to natural and artificial light sources and a 10 MeV electron beam source. Oxidation products from the ring structure were separated by gas chromatography (GC) and characterized using GC-MS spectroscopy. Chromatographic data for some of the products are reported for the first time.

MATERIALS AND METHODS

Materials and Reagents. Sunflower, olive, rapeseed, and soybean oils were of edible quality and purchased at a local supermarket. 5α-Cholestan-3β-ol (cholestanol, 95%) and cholest-5-ene-3β,19-diol (19-hydroxycholesterol, 95%) were purchased from Sigma (Steinheim, Germany) and from Steraloids (Newport, RI), respectively, and were used as internal standards. β-Sitosterol oxides, 24α-ethylcholest-5-ene-3β,7α-diol (7α-hydroxysitosterol), 24α-ethylcholest-5-ene-3β,7β-diol (7β-hydroxysitosterol), 24α-ethylcholestan-5α,6α-epoxy-3β-ol (5,6α-epoxysitosterol), 24α-ethylcholestan-5β,6β-epoxy-3β-ol (5,6β-epoxysitosterol), 24α-ethylcholest-5-en-3β-ol-7-one (7-ketositosterol), and 24α-ethylcholestane-3β,5α,6β-triol (5α,6β-dihydroxysitosterol), were chemically synthesized in the laboratory (*31*).

Reagents, of analytical grade, were purchased as follows: ethanol from Carlo Erba (Val de Reuil, France), cyclohexane from Fluka (Buchs, Switzerland), diethyl ether from Riedel-de Haën (Seelze, Germany), acetone and pyridine from Prolabo (Fontenay-sous-Bois, France), isooctane and potassium hydroxide pellets from Merck (Darmstadt, Germany), anhydrous sodium sulfate (Na_2SO_4) from SDS (Peypin, France), and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) from Sigma-Aldrich. Solid phase extraction (SPE) silica gel cartridges (3 mL/500 mg) were purchased from Macherey-Nagel (Chromabond, Düren, Germany).

Preparation of Oil Samples and Radiation Treatments. Prior to the assays, oil samples (1.5 g) were separately weighed into glass dishes (10 mm × 60 mm i.d.), which were immediately closed with glass covers. These covers were removed just before treatment, to ensure a uniform exposure of the samples to air and light.

Sunlight radiation was carried out by exposing oil samples to natural solar light (May, 2005, in Illkirch, France) behind a glass window at room temperature for 10, 20, and 30 days. A high-pressure Hg lamp HBO 200 W fitted with IR quartz and glass filters (sun test, Critt Matériaux, Schiltigheim, France) was used in order to reproduce the sunlight spectrum but with a higher intensity. The oil dishes without glass covers were exposed for 21, 42, and 63 h at a distance of 15 cm from the lamp, which represented a light intensity of 765 W/m^2 (300−800 nm). Under these conditions, a 63 h exposure period corresponded to a month long exposure to natural sunlight in Europe.

Irradiation treatments were carried out with an electron beam accelerator (CIRCE III Linear Electron Accelerator, MeV Industrie S. A., Jouy-en-Josas, France) located in the Bundesforschungsanstalt für Ernährung und Lebensmittel (Karlsruhe, Germany). The three absorbed doses (0.93, 2.69, and 9.30 kGy) were controlled by FWT 60-00 radiachromic dosimeters (Far West Technology, Goleta, CA) calibrated against alanine dosimeters (Laboratoire National Henri Becquerel, Gif-sur-Yvette, France), which are the French national reference for high-absorbed doses. Treatments were performed at 6−8 °C. Oil samples were also kept in the dark without irradiation as controls.

The oil samples were analyzed immediately after exposure to the UV and sunlight sources. After the electron beam irradiations, oil samples were immediately covered and stored in the dark at 4 °C, and then analysis of oxides in oils was carried out 1 week later. Prior to analysis, homogenized samples (200 mg, $n = 3$) were weighed into 30 mL brown glass vials.

Analysis of Phytosterol Oxides in Oil Samples. Extraction of phytosterol oxides and GC-MS analysis was carried out as previously described (*30*), taking advantage of the previously synthesized standards of phytosterol oxides (*31*). Briefly, a 200 mg oil sample was spiked with a 19-hydroxycholesterol solution (20 μL, 1 mg/mL in ethyl acetate) as an internal standard. After removal of the solvent with a gentle flow of nitrogen, the sample was dissolved in a mixture of ethanol (9 mL) and a saturated aqueous KOH solution (0.5 mL) before it was submitted to an overnight (15 h) soft saponification at room temperature. The unsaponifiable fraction was extracted with diethyl ether, and the oxides were separated from the matrix using a SPE silica gel cartridge and converted to trimethylsilyl (TMS) ethers with pyridine (50 μL) and MSTFA (40 μL). Extraction steps were done in brown bottles and at room temperature. Analysis of 1 μL samples was carried out using a GC-MS chromatograph (capillary column VF-5 ms, stationary phase 5% phenyl−95% dimethylpolysiloxane, thickness of 0.1 μm, 60 m × 0.25 mm, Varian, France). The chromatograms were recorded in the total ion current (TIC) mode, and the quantification was done with the reconstructed ion trace chromatogram of the ions m/z 356 + 353 + 485 + 413 + 395 + 432 + 396.

Statistical Analysis. All analyses were performed in triplicate. Comparative data analysis was done by a two-sided Student test within a 95% confidence interval, after the equality of the standard deviations by a two-sided Fischer−Snedecor test within a 95% confidence interval was verified.

The Student coefficient t was computed

$$t = \frac{|m_1 - m_2|}{\sqrt{\left(\frac{\nu_1 s_1^{\,2} + \nu_2 s_2^{\,2}}{\nu_1 + \nu_2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where m_1 and m_2 were the average measured amounts, s_1 and s_2 were the standard deviations, ν_1 and ν_2 were the degrees of freedom, and n_1 and n_2 were numbers of measures. This coefficient was then compared to the Student coefficient $t_{(\nu,0.975)}$ (*32*).

RESULTS AND DISCUSSION

Chromatographic separations of sterols and their oxides were achieved on an apolar VF-5 ms capillary column and identified by mass spectroscopic analysis. The initial contents of β-sitosterol were 2337, 1690, 1320, and 3541 mg/kg in sunflower, soybean, olive, and rapeseed oils, respectively (*33*). **Figure 1** shows typical GC chromatograms of phytosterol oxides generated in oils under artificial light with TIC detection mode and the reconstructed ion trace chromatogram of the ions m/z 356 + 353 + 485 + 413 + 395 + 432 + 396. Some β-sitosterol oxides were originally present in native sunflower, soybean, and rapeseed oils that we used (**Table 1**). In sunflower oil, 7α-hydroxy (2.3 μg/g), 7β-hydroxy (2.5 μg/g), and 7-ketositosterol (4.8 μg/g) were detected. Trace amounts of 7α-hydroxy (0.3 μg/g) and 7β-hydroxysitosterol (0.5 μg/g) were recovered from native soybean oils. Three β-sitosterol oxides were also found at low concentrations in rapeseed oil. These oxides may be formed during oil processing and refinement (*34*, *35*). However, no detectable levels of sterol oxides were found in native olive oil.

Tables 1−3 show the amounts of β-sitosterol oxides formed in the investigated oils after exposure to sunlight, artificial UV light, and electron beam radiations for various durations. With sunlight, total β-sitosterol oxides contents rapidly increased during the first 20 days in all studied oils, reaching amounts of 1156, 1070, 648, and 2421 μg/g in sunflower, soybean, olive, and rapeseed oils, respectively (**Table 1**). However, after 20

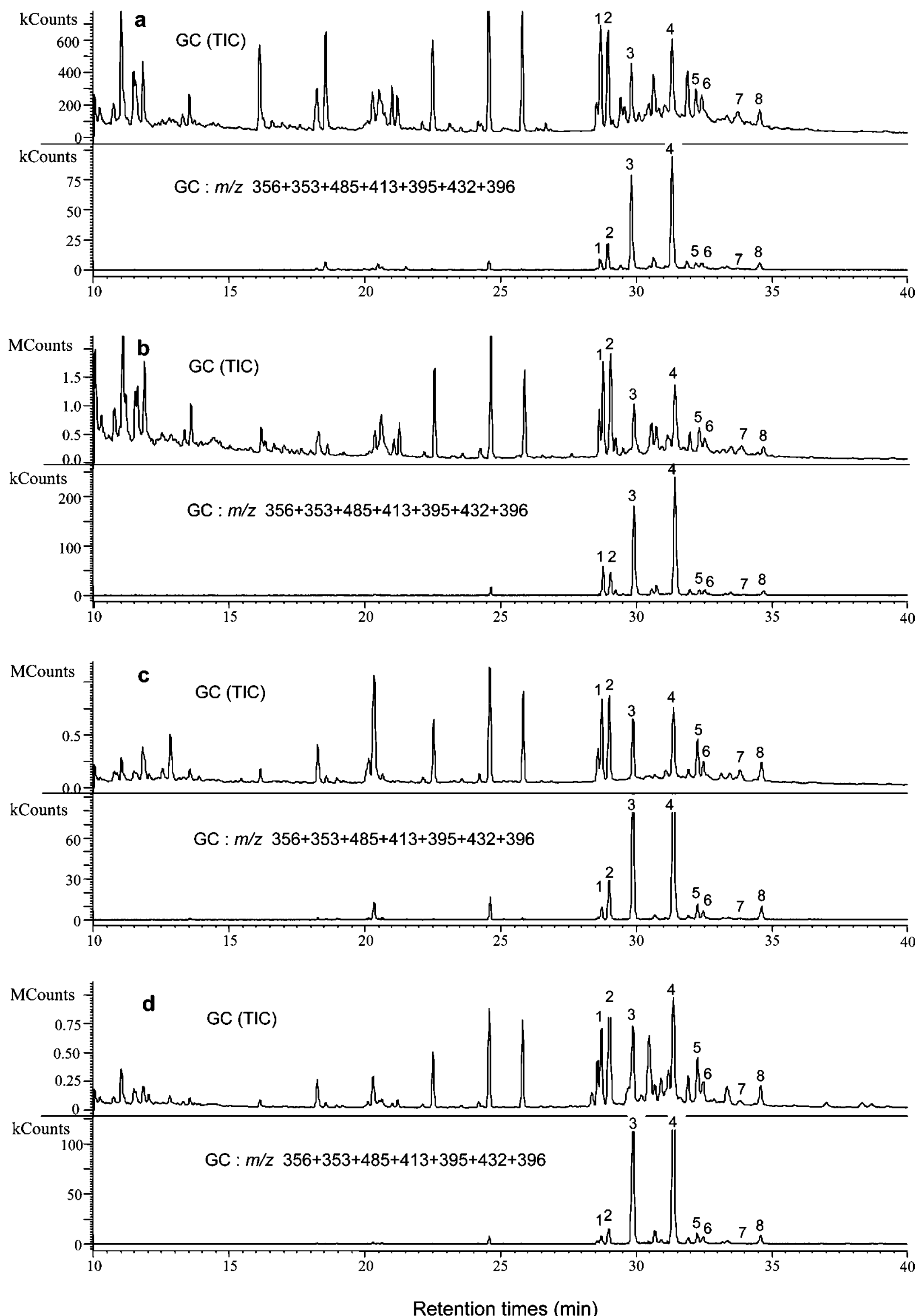

Figure 1. Typical GC chromatograms (TIC mode and reconstructed ion trace chromtogram, *m/z* 356 + 353 + 485 + 413 + 395 + 432 + 396) obtained by GC-MS analysis of the polar fractions of the exposed oil samples: (**a**) sunflower oil, (**b**) soybean oil, (**c**) olive oil, and (**d**) rapeseed oil. Chromatographic peaks: 1, cholestanol (internal standard); 2, 19-hydroxycholesterol (internal standard); 3, 7α-hydroxysitosterol; 4, 7β-hydroxysitosterol; 5, 5,6β-epoxysitosterol; 6, 5,6α-epoxysitosterol; 7, 5α,6β-dihydroxysitosterol; and 8, 7-ketositosterol.

days of sunlight exposure, sunflower and soybean oils have changed to a gellike texture, and the amounts of the three major oxides (7α-hydroxy, 7β-hydroxy, and 7-ketositosterol) started decreasing, while other oxides were still being formed. This decrease might be due to the higher oil viscosity, which would reduce the mobility of oxygen, thereby hindering the oxidative degradation of phytosterols in oils. It is also probable that these oxidation products are prone to further decomposition as already observed for fatty acid hydroperoxide. In olive oil, only the content of 7-ketositosterol decreased after 20 days, whereas the production of other oxides was only slowed. The oxides in rapeseed oil steadily increased under sunlight during the 30 days exposure period, and the total amount of the six β-sitosterol oxides was the highest among all four oils samples studied. It

Table 1. Concentrations (μg/g) of β-Sitosterol Oxides in Various Oils under Sunlight[a]

oil samples	exposure time (days)	7α-hydroxy-sitosterol	7β-hydroxy-sitosterol	5,6β-epoxy-sitosterol	5,6α-epoxy-sitosterol	5α,6β-dihydroxy-sitosterol	7-keto-sitosterol	total amount (μg/g)	% of β-sitosterol oxidized
sunflower oil	0	2.3 ± 0.1	2.5 ± 0.0	<0.1	<0.1	<0.1	4.8 ± 0.3	9.6	<0.4
	10	169 ± 3	238 ± 4	55 ± 5	38 ± 3	9.3 ± 0.4	124 ± 5	633	9
	20	237 ± 6	310.3 ± 0.4	206 ± 3	138 ± 1	15.6 ± 0.6	249.3 ± 0.3	1156	45
	30	183 ± 2	188 ± 2	215 ± 1	164 ± 4	50.1 ± 0.9	206 ± 2	1007	39
soybean oil	0	0.3 ± 0.1	0.5 ± 0.0	<0.1	<0.1	<0.1	<0.1	0.8	<0.1
	10	94 ± 4	146.7 ± 0.5	57 ± 1	23 ± 2	4.9 ± 0.4	92 ± 1	417	22
	20	192 ± 2	215 ± 2	157 ± 4	112 ± 1	9.8 ± 0.7	192 ± 3	1070	50
	30	159 ± 2	180 ± 2	207 ± 2	126 ± 2	30.0 ± 0.4	192 ± 1	895	49
olive oil	0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	10	78 ± 1	893 ± 1	29 ± 2	14 ± 3	2.1 ± 0.9	84 ± 6	297	20
	20	148 ± 3	187 ± 1	77 ± 3	56 ± 1	4.3 ± 0.5	176 ± 3	648	44
	30	164 ± 2	203 ± 2	106 ± 3	71 ± 1	5.8 ± 0.2	127 ± 2	676	46
rapeseed oil	0	0.2 ± 0.0	0.3 ± 0.0	<0.1	<0.1	<0.1	1.4 ± 0.3	1.9	<0.1
	10	175.8 ± 0.2	240 ± 2	64 ± 2	47.6 ± 0.1	8.9 ± 0.6	122 ± 1	659	17
	20	292 ± 2	396 ± 3	242 ± 0.4	142 ± 1	16.4 ± 0.3	239 ± 1	1327	34
	30	526.1 ± 0.8	651 ± 2	535 ± 2	280 ± 2	34.3 ± 0.2	395 ± 2	2421	62

[a] Mean ± standard deviation ($n = 3$).

Table 2. Concentrations (μg/g) of β-Sitosterol Oxides in Various Oils under UV Light[a]

oil samples	exposure time (h)	7α-hydroxy-sitosterol	7β-hydroxy-sitosterol	5,6β-epoxy-sitosterol	5,6α-epoxy-sitosterol	5α,6β-dihydroxy-sitosterol	7-keto-sitosterol	total amount (μg/g)	% of β-sitosterol oxidized
sunflower oil	0	2.3 ± 0.1	2.5 ± 0.0	<0.1	<0.1	<0.1	4.8 ± 0.3	9.6	0.4
	21	31.5 ± 0.3	46 ± 3	22 ± 1	9 ± 1	<0.1	24.2 ± 0.5	132	5
	42	54 ± 2	80 ± 1	48 ± 0.7	20.1 ± 0.4	2.8 ± 0.2	26 ± 1	231	9
	63	84 ± 2	117 ± 2	78 ± 2	40 ± 3	4.3 ± 0.0	31 ± 2	354	14
soybean oil	0	0.3 ± 0.1	0.5 ± 0.0	<0.1	<0.1	<0.1	<0.1	0.8	0.1
	21	26.8 ± 1.0	40 ± 1	18.3 ± 0.6	<0.1	0.7 ± 0.1	18.5 ± 0.7	104	6
	42	55.4 ± 0.8	70 ± 2	38 ± 1	19 ± 2	1.8 ± 0.5	22.8 ± 0.0	207	11
	63	97 ± 4	89 ± 1	81 ± 3	36.2 ± 0.5	2.8 ± 0.4	25.5 ± 0.8	331	18
olive oil	0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	21	20.7 ± 0.5	29.5 ± 0.6	21.3 ± 0.1	7.1 ± 0.3	<0.1	23.3 ± 0.6	102	7
	42	50 ± 2	64 ± 2	64 ± 1	24 ± 3	0.9 ± 0.0	30.0 ± 0.9	233	16
	63	66 ± 2	77.1 ± 0.5	87 ± 3	37.7 ± 1.0	1.7 ± 0.1	23.7 ± 0.4	294	17
rapeseed oil	0	0.2 ± 0.0	0.3 ± 0.0	<0.1	<0.1	<0.1	1.4 ± 0.3	1.9	0.1
	21	114.8 ± 0.1	174.3 ± 1.0	116 ± 1	41.6 ± 0.6	4.3 ± 0.6	71.2 ± 0.5	522	12
	42	141 ± 2	216 ± 3	136 ± 4	60 ± 2	4.8 ± 0.6	68.1 ± 0.4	626	16
	63	242 ± 5	340 ± 2	308 ± 2	111 ± 2	9.0 ± 1.0	72.2 ± 0.7	1082	28

[a] Mean ± standard deviation ($n = 3$).

Table 3. Concentrations (μg/g) of β-Sitosterol Oxides in Various Oils Exposed to a 10 MeV Electron Beam Radiation[a]

oil samples	irridiation doses (kGy)	7α-hydroxy-sitosterol	7β-hydroxy-sitosterol	5,6β-epoxy-sitosterol	5,6α-epoxy-sitosterol	5α,6β-dihydroxy-sitosterol	7-keto-sitosterol	total amount (μg/g)	% of β-sitosterol oxidized
sunflower oil	0	2.3 ± 0.1	2.5 ± 0.0	<0.1	<0.1	0	4.8 ± 0.3	9.6	0.4
	0.93	4.3 ± 0.2	5.1 ± 0.3	<0.1	<0.1	1.5 ± 0.3	10.0 ± 0.2	20.9	0.8
	2.69	4.7 ± 0.1	6.4 ± 0.2	<0.1	<0.1	1.6 ± 0.2	10.7 ± 0.4	23.4	0.9
	9.30	4.7 ± 0.2	6.4 ± 0.3	<0.1	<0.1	1.7 ± 0.1	10.9 ± 0.5	23.7	0.9
soybean oil	0	0.3 ± 0.1	0.5 ± 0.0	<0.1	<0.1	<0.1	<0.1	0.8	0.1
	0.93	0.3 ± 0.0	0.6 ± 0.0	<0.1	<0.1	1.0 ± 0.1	0.9 ± 0.3	2.8	0.1
	2.69	0.4 ± 0.0	0.6 ± 0.1	<0.1	<0.1	1.2 ± 0.3	1.3 ± 0.1	3.5	0.2
	9.30	0.6 ± 0.1	0.8 ± 0.1	<0.1	<0.1	1.3 ± 0.0	1.7 ± 0.0	4.4	0.2
olive oil	0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	0.93	0.5 ± 0.0	1.1 ± 0.1	<0.1	<0.1	<0.1	<0.1	1.6	0.1
	2.69	0.6 ± 0.1	1.6 ± 0.2	<0.1	<0.1	<0.1	<0.1	2.2	0.1
	9.30	0.7 ± 0.1	1.5 ± 0.4	<0.1	<0.1	<0.1	0.6 ± 0.0	2.8	0.1
rapeseed oil	0	0.2 ± 0.0	0.3 ± 0.0	<0.1	<0.1	<0.1	1.4 ± 0.3	1.9	0.1
	0.93	0.4 ± 0.1	0.6 ± 0.1	<0.1	<0.1	0.6 ± 0.1	0.9 ± 0.0	2.5	0.1
	2.69	0.4 ± 0.1	0.8 ± 0.2	<0.1	<0.1	0.6 ± 0.0	0.8 ± 0.0	2.6	0.1
	9.30	0.4 ± 0.1	0.7 ± 0.1	<0.1	<0.1	0.6 ± 0.0	1.0 ± 0.0	2.7	0.1

[a] Mean ± standard deviation ($n = 3$).

has been shown that epoxy-sterols may be formed by reaction between triglyceridic hydroperoxides and sterols (*36*) and that diols are formed by hydratation of epoxyde. Therefore, theses oxidation products may be formed not only by the direct reaction of molecular oxygen and sterol but also through the action of fatty acids and water, impurities present in the sample. This is consistent with the various concentrations of the different oxides observed in the different oils.

With UV light, used to mimic the sunlight, except for 7-ketositosterol, contents of β-sitosterol oxides steadily increased

with exposure (**Table 2**). The same major oxides, 7α-hydroxy, 7β-hydroxy, and 5,6β-epoxysitosterol, were formed in all four oils samples studied. As with sunlight, the amount of oxides generated in rapeseed oil was higher than in other oils. However, much lower (about half) amounts of phytosterol oxides were obtained with comparison to sunlight. This is most certainly due to the fact that sunlight trials were performed over a longer period. The very low rate of oxidation allowed most dissolved oxygen to react with phytosterols and the surrounding atmospheric oxygen to diffuse into the oils and produce more oxyphytosterols. A slight limitation of the present study is the lack of having control oils shielded from light (but not from oxygen) and analyzed throughout the trials. It would certainly have made a more sound experiment and pointed out the slight effect of the sole oxygen, without photoinitiation, at room temperature (*30*).

In addition to electromagnetic waves such as solar radiations, oil-based products may be exposed to other high-energy radiation sources susceptible to inducing oxidation phenomena of sterols, namely, food irradiation sources. As mentioned above, radiation sources, such as high-energy electron beams (energy below 10 MeV) or ^{60}Co sources (energy of 1.17 and 1.33 MeV), are used in the food industry to ensure food safety and hygiene. Indeed, these radiations efficiently kill the pathogenic and spoilage microflora. Plant foods (fruits, tubers, bulbs, cereals, legumes, aromatic herbs, and seasonings) are among the products most often subjected to food irradiation. Their irradiation is aimed at reducing postharvest losses due to infestations by insects, increasing the shelf life of fruits and vegetables, inhibiting sprout and germination of bulbs and tubers, and ensuring the safety of plant-based ready-to-eat meals. The latter often contain vegetable oils with or without phytosterol supplementation.

In this study, oil exposure to electron beam radiation had little effect on the formation of oxides. Epoxy α- and β-oxides, which were predominant with light sources, were absolutely not affected by electron beam radiation (**Table 3**). Overall, oxide amounts detected in oils before and after irradiation were not significantly different, even at the highest absorbed dose of 9.30 kGy, which almost corresponds to the maximum authorized treatment in Europe (10 kGy). A simple explanation of this poor yield in production of β-sitosterol oxides by electron beam irradiation would be the very high dose rate obtained with the modern irradiation plants (several tens of kGy/s). An absorbed dose of about 9.30 kGy only requires one fraction of a second and available oxygen for the oxidative reactions is immediately consumed locally, while the surrounding atmospheric oxygen has no time to diffuse into the food sample and take part in the oxidative process. This result is very consistent with the observed trend with sunlight and UV light: the higher the dose rate used (the shorter the exposition), the lower the amounts of oxides formed.

The present work indicated that UV radiation was able to induce phytosterol peroxidation, while an electron beam had little oxidizing effect due to the higher dose rate used. The data reported here might be useful to the food industry and the consumer in order to take steps aimed at limiting the formation of sterol oxides.

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