REVIEW

Phytosterol oxidation products in enriched foods: Occurrence, exposure, and biological effects

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Hypercholesterolemia is an important risk factor for the development of cardiovascular diseases. Dietary intake of phytosterols/phytostanols and their fatty acid esters results in a reduction of the LDL and total plasma cholesterol levels. Therefore, these constituents are added to a broad spectrum of foods. As in the case of cholesterol, thermo-oxidative treatment of phytosterols may result in the formation of phytosterol oxidation products (POPs), i.e. keto-, hydroxy-, and epoxy-derivatives. This review summarizes and evaluates the current knowledge regarding POPs in the light of the potentially increasing dietary exposure to these constituents via the consumption of foods enriched with phytosterols/phytostanols and their esters. Data on the occurrence of POPs and approaches to assess the potential intake of POPs resulting from the consumption of enriched foods are described. The knowledge on the uptake of POPs and the presently available data on the impact of the consumption of enriched foods on the levels of POPs in humans are discussed. Biological effects of POPs, such as potential proatherogenic properties or the loss of the cholesterol-lowering effects compared to nonoxidized phytosterols, are discussed. Finally, knowledge gaps are outlined and recommendations for further research needed for a safety assessment of POPs are presented.

Keywords:
Enriched foods / Intake assessment / Phytosterol oxidation products / Research needs / Safety assessment

1 Introduction

Hypercholesterolemia is an important risk factor for the development of cardiovascular diseases. A daily dietary intake of 2 g phytosterols/phytostanols results in a reduction of LDL and total plasma cholesterol levels of approximately 10% [1–3]. Owing to these cholesterol-lowering properties, phytosterols/phytostanols and their fatty acid esters were among the first ingredients used to enrich foods and thus to obtain an additional beneficial effect. On the basis of a safety assessment by the Scientific Committee on Food (SCF) in 2000 (http://ec.europa.eu/food/fs/sc/scf/out56_en.pdf; accessed: Apr 22, 2014), a yellow fat spread enriched with specified amounts of phytosteryl fatty acid esters was the first product authorized by the EU Commission as a novel food according to Regulation (EC) No 258/97 [4, 5]. In the meantime, a broad spectrum of other foods with added phytosteryl/phytostanyl fatty acid esters has been placed on the market in the European Union. They comprise milk-type products, yoghurt-type products, milk-based fruit drinks, soy-based drinks, cheese-type products, salad dressings, spice sauces, rye bread, rice drinks, and oils [6]; regularly updated lists of the respective authorizations (http://www.bfr.bund.de/cm/343/140417-antraege-auf-zulassung-neuartiger-lebensmittel-gemaess-artikel-4-der-verordnung-eg-nr-258-97.pdf; accessed: Oct 16, 20014) and notifications (http://www.bfr.bund.de/cm/343/notifizierungen-neuartiger-lebensmittel-gemaess-artikel-5-der-verordnung-eg-258-97.pdf; accessed: Oct 16, 2014) are available. Phytosterols/phytostanols and their fatty acid esters are among those food ingredients for which health claims referring to the reduction of disease risk have been permitted [7, 8].

There is no evidence of additional cholesterol-lowering benefits at intakes of phytosterols higher than 3 g/day [3];

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Abbreviations: **ABCG5**, ATP-binding cassette transporter G5; **ABCG8**, ATP-binding cassette transporter G8; **ACh**, acetylcholine; **Apc**, Adenomatous polyposis coli; **DFG**, German Research Council; **IL**, interleukin; **PLM**, postlaunch monitoring; **POPs**, phytosterol oxidation products; **SCF**, Scientific Committee on Foods; **SKLM**, Senate Commission on Food Safety; **TNFα**, tumor necrosis factor α

in addition, high intakes may induce undesirable effects, such as a reduction of plasma levels of ß-carotene [1, 9, 10]. Therefore, in its general view on the long-term effects of the intake of elevated levels of phytosterols from multiple dietary sources, the SCF considered it prudent to avoid intakes exceeding a range of 1–3 g/day (http://ec.europa.eu/food/fs/sc/scf/out143_en.pdf; accessed: Oct 16, 2014). This precautionary limit is in line with the group acceptable daily intake of 0–40 mg/kg bw for the group of phytosterols, phytostanols, and their esters, expressed as the sum of phytosterols and phytostanols in their free form, later derived by Joint FAO/WHO Expert Committee on Food Additives [11].

The Senate Commission on Food Safety (SKLM) of the German Research Council (DFG) previously published two scientific opinions regarding the use of phytosteryl/phytostanyl fatty acid esters in foods in 2001 (http://www.dfg.de/download/pdf/dfg_im_profil/reden_stellungnahmen/2003/sklm_phytosterole21092001.pdf; accessed: Oct 16, 2014) and 2007 (http://www.dfg.de/download/pdf/dfg_im_profil/reden_stellungnahmen/2007/sklm_phytosterine_07.pdf; accessed: Oct 16, 2014). They focused on the need for assessment of individual phytosteryl/phytostanyl ester preparations and the importance of the corresponding specifications. In addition, they drew particular attention to the challenges arising from the broad spectrum of enriched food categories and the uncertainties in ensuring that an intake of 1–3 g/day is not exceeded. The need for current and reliable consumption data and for measures to ensure that the products are only consumed by the target groups was emphasized.

In order to allow users to restrict their consumption to a maximum of 3 g of phytosterols/phytostanols per day and to ensure that the product reaches its target group, specific provisions regarding the labeling of foods and food ingredients with added phytosterols/phytostanols and their esters have been implemented; for example, a label is required that indicates that the consumption of more than 3 g/day of added plant sterols/stanols should be avoided [12]. However, a consumer awareness study performed in Germany revealed that 45% of the consumers did not belong to the target group, 3.5% were children and only 1% were aware that an intake of 3 g phytosterols/day should not be exceeded [13]. Data on the actual exposure of consumers to phytosterols via the multiple sources of enriched foods are also inconsistent. According to a postlaunch monitoring (PLM) survey on consumer purchases of foods (spreads, salad dressings, milk-, and yoghurt-type products) with added phytosterols in five European countries, the mean phytosterol intakes per household were 0.35–0.86 g/day. In the 95th percentile of the population, intakes ranged from 1.0 g/day in France to 3.7 g/day in The Netherlands; The Netherlands was the only country in which approximately 6% of households were identified as potential "over consumers" [14]. These data indicating that overconsumption of phytosterols seems unlikely are in agreement with the results obtained in the mandatory PLM performed by Unilever covering the first year of marketing of enriched vegetable oil spreads; in that survey the median intakes of phytosterols for regular purchasers were 1.2–1.4 g/day, the 95th percentile intakes ranged from 2.2 g/day in France to 3.6 g/day in The Netherlands [15]. On the other hand, a significantly higher mean phytosterol intake (2.45 g/day) was reported in a study performed on the Irish market. In total, 23% of the respondents had mean phytosterol intakes higher than 3 g/day and the majority of consumers (58%) had been consuming these products for more than one year [16]. A study by Sioen et al. (2011) investigating the consumption of phytosterol-enriched foods in Belgium also identified 16% of consumers to have a phytosterol intake above 3 g/day [17].

Another controversial issue is the increased absorption of phytosterols, potentially resulting in their accumulation and subsequently in the promotion of vascular diseases [18]. Examples are the presence of plant sterols in atherosclerotic plaques of patients undergoing carotid endarterectomy [19], the accumulation of plant sterols in human stenotic aortic valves [20, 21], and the effects of long-term plant sterol and stanol consumption on the retinal vasculature [22]. The potential accumulation of phytosterols in the arterial wall versus the plasma cholesterol-reducing effect of dietary phytosterols/phytostanols and, thus, their usefulness in preventing coronary heart disease are being intensively discussed [23–26]. One recent study showed the induction of intestinal adenoma formation in Apc^{Min} (*Adenomatous polyposis coli*) mice having been fed a plant stanol-enriched diet [27].

Phytosterols/phytostanols are structurally very similar to cholesterol. This similarity is the molecular basis for the cholesterol-lowering properties of these substances. However, undesirable reactions known for cholesterol may also be expected in the case of phytosterols. A typical example is the formation of so-called cholesterol oxidation products, i.e. keto-, hydroxy-, and epoxy derivatives of cholesterol, a well-known class of substances studied in detail for many years. On the one hand, cholesterol oxidation products are crucial intermediates in mammalian metabolism, are enzymatically synthesized in vivo, and serve several regulatory purposes such as cholesterol homeostasis [28, 29]. On the other hand, they may be formed endogenously via nonenzymatic oxidation of cholesterol and may also be absorbed from the diet. In cholesterol-containing foods, cholesterol oxidation products can be formed via processing and storage [29]. Elevated plasma levels of cholesterol oxidation products have particularly been correlated to atherogenic effects, and are also thought to be involved in other inflammatory processes such as neurodegeneration [30]. Therefore, the occurrence in foods and the subsequent dietary intake not only of intact cholesterol but also of cholesterol oxidation products has been in the focus of recent research activities. An increased intake of dietary cholesterol oxidation products was shown to be associated with impaired hepatic function and lipid metabolism and ultimately atherosclerotic progression in various animal models [31–39].

Taking into account the structural similarities between cholesterol and phytosterol oxidation products (POPs)

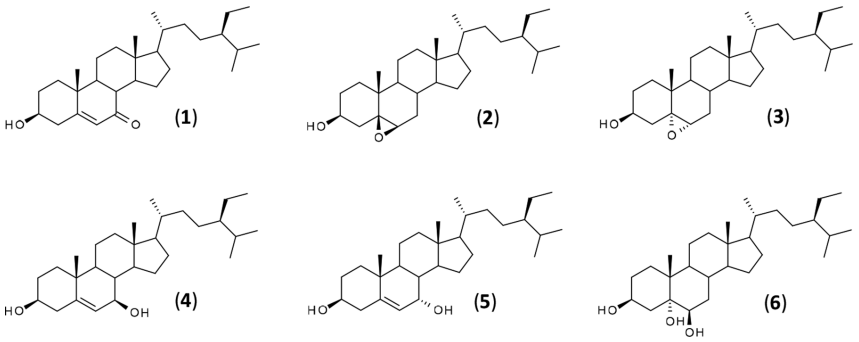

Figure 1. Structures of phytosterol oxidation products derived from ß-sitosterol: 7-keto-sitosterol (**1**), 5,6ß-epoxy-sitosterol (**2**), 5,6α-epoxy-sitosterol (**3**), 7ß-hydroxy-sitosterol (**4**), 7α-hydroxy-sitosterol (**5**), sitostanetriol (**6**).

and considering the growing number and the variety of products enriched with phytosteryl/phytostanyl fatty acid esters, studies on the formation and intake of POPs and the assessment of their potential adverse effects seem advisable. A present application to extend the use of phytosteryl esters to spreads and liquid vegetable fat-based emulsions specifically intended for cooking and baking underlines the relevance of such an evaluation (http://acnfp.food.gov.uk/assess/fullapplics/phytosterol; accessed Oct 16, 2014).

In the last decade, research activities regarding POPs significantly increased; the progress has been reflected in a series of reviews [30, 40–45]. The objective of this review is to summarize and to evaluate the current knowledge, particularly in the light of the potentially increasing dietary exposure to POPs via the consumption of foods enriched with phytosterols/phytostanols and their esters, and to outline research needs for a safety assessment.

2 Analytical approaches

Thermooxidation of phytosterols occurring in foods encompasses a sequence of reactions resulting in primary (hydroperoxides), secondary (polar: ketones, alcohols, epoxides; unpolar: steradienes, steratrienes), and tertiary oxidation products (dimers, oligomers, polymers). Owing to the analytical capabilities, the focus has almost exclusively been put on the secondary polar POPs. Accordingly, the term "POPs" used in this opinion refers to the keto-, epoxy-, and hydroxy-compounds derived from the respective sterols/stanols; Fig. 1 shows examples of structures of POPs obtained from ß-sitosterol.

There are various analytical methodologies available based on (i) lipid extraction and saponification or transesterification, (ii) isolation and purification via thin layer chromatography or solid phase extraction, (iii) derivatization to trimethylsilyl ethers, and (iv) detection via HPLC- and GC-based techniques [46, 47].

These methods have been employed to generate a broad spectrum of analytical data on model systems involving the thermal treatment of phytosterol standards [48–50]. Thermooxidations under different time/temperature conditions revealed that some of the secondary POPs constitute intermediates, which are further transformed or degraded in the course of the reaction [51]. First attempts to isolate fractions containing dimers, trimers, and tetramers via size exclusion chromatography have been described, and structures for sterol dimers have been proposed [52–56].

Heating of stigmasterol for 3 h at 180°C resulted in a loss of the intact sterol of 61%. Polar, midpolar and nonpolar oxidation products accounted for 39% of this loss; the formation of dimers and polymers accounted for 30% of the loss. This means that there is a gap in the mass balance, leaving 31% of the stigmasterol loss unexplained [57].

There are data available indicating qualitative and quantitative differences in oxidation profiles between free and esterified phytosterols [58–63]. Complex mixtures are to be expected owing to potential oxidations in the sterol as well as in the fatty acid moiety of phytosteryl esters of unsaturated fatty acids [64]. Very recent studies described approaches to analyze intact oxidized phytosteryl fatty acid esters via HPLC-ESI-MS [65].

3 Occurrence in foods

3.1 Nonenriched foods

Data on POPs exist for various foods containing phytosterols/phytostanols or their esters as naturally occurring constituents. The presence of POPs in crude vegetable oils and their fate during refining has been analyzed [66]. The effects of the heating of vegetable oils on the formation of POPs have been studied in model experiments [48, 67] as well as under industrial frying conditions [68]. Commercial potato crisps [69], potato chips prepared in different vegetable oils [70], and French fries prepared in these oils [68] have been investigated. Sterol oxidation in infant milk formulas and milk cereals [71] and in ready-to-eat infant foods during storage [72] have also been studied. As examples, data on spread [73], French fries [68], and potato crisps [69] are summarized in Table 1. The average content of POPs in the heat-treated products French fries and potato crisps was around 1 mg/kg (except for the French fries obtained as restaurant samples); this content corresponds to an oxidation rate of approximately 0.8%.

3.2 Enriched foods

Information on the contents of POPs in foods enriched with phytosteryl/phytostanyl fatty acid esters is limited; data

Table 1. Contents of phytosterol oxidation products (POPs) determined in selected nonenriched foods and resulting intakes calculated on the basis of consumption data of the corresponding foods

Type of food	POPs [mg/kg]	Oxidation rate[%][a)]	POP intake [mg/day][b)]		Ref.
			Median	95th percentile	
Spread					
63% fat	13.3	0.41	0.14[c)]	0.65[c)]	[73]
French Fries					
Oven, 225°C, 15 min	1.2	0.5	0.08	0.19	[68]
Prefried samples	0.8	1.3	0.06	0.12	[68]
Restaurant samples	3.4	0.8	0.23	0.53	[68]
Potato crisps					
High fat (>25%)	1.1	0.6	0.02	0.06	[69]
Low fat (<25%)	1.2	0.8	0.03	0.06	[69]

a) Calculated as percentage of POPs with respect to the initial phytosterol content.
b) Calculated on the basis of consumption data for adults among consumers only across European countries (http://www.efsa.europa.eu/de/consultationsclosed/call/140701.pdf; accessed Oct 16, 2014), except for the data labelled by footnote [c)].
c) Calculated on the basis of consumption data for adults among consumers only in Germany (http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm; accessed Oct 16, 2014).

available for the matrices milk, spreads, liquid spread for cooking and baking, and chocolate are summarized in Table 2. The analyzed products differ regarding type (phytosteryl versus phytostanyl esters), degree of enrichment and employed treatments (heat, storage).

The contents of POPs determined in conventionally pasteurized milk enriched with free phytosterols or phytosteryl esters were consistently around 2 mg/kg, corresponding to oxidation rates between 0.05 and 0.07%. The effect of thermal processing on the formation of POPs in milk was investigated using different heating techniques [74]. The detected contents ranged from 3.5 to 6.4 mg/kg; microwave heating at 900 W for 1.5 minutes yielded the highest amounts of POPs. However, the amounts of POPs and the corresponding

Table 2. Contents of phytosterol oxidation products (POPs) determined in enriched foods and resulting intakes calculated on the basis of a consumption of enriched foods corresponding to 3 g phytosterols per day

Type of food	Treatment	POPs [mg/kg]	Oxidation rate [%][a)]	POP intake [mg/day][b)]	Ref.
Milk					
Free phytosterols (≙ 0.5% phytosterols)	Pasteurization (127°C, 2 s)	2.2	0.05	1.32	[76]
Phytosteryl esters (≙ 0.5% phytosterols)	Pasteurization (127°C, 2 s)	2.0	0.04	1.2	[76]
Phytostanyl esters (≙ 0.5% phytostanols)	Pasteurization (127°C, 2 s)	0.2	0.004	0.1	[76]
Phytosteryl esters (≙ 0.3% phytosterols)	Pasteurization	2.2	0.07	2.2	[74]
Phytosteryl esters (≙ 0.3% phytosterols)	65°C, 24 h	3.5	0.1	3.5	[74]
Phytosteryl esters (≙ 0.3% phytosterols)	Microwave (900 W, 1.5 min)	6.4	0.21	6.4	[74]
Phytosteryl esters (≙ 0.3% phytosterols)	Microwave (900 W, 2.0 min)	5.6	0.19	5.6	[74]
Phytosteryl esters (≙ 0.3% phytosterols)	Electric heating (15 min)	4.2	0.14	4.2	[74]
Spread					
Phytosteryl esters (≙ 8% phytosterols)	—	68	0.09	2.6	[75]
Phytosteryl esters (≙ 6% phytosterols)	—	46.5	0.07	2.3	[73]
Phytosteryl esters	—	12	—	—	[67]
Phytostanyl esters	—	255	—	9.6	[79]
Phytostanyl esters	Storage (6 weeks, 4°C)	354	0.12	13.3	[79]
Phytostanyl esters	Storage (6 weeks, 20°C)	734	0.61	27.5	[79]
Liquid spread					
Phytosteryl esters (≙ 7.5% phytosterols)	Heating (205°C, 30 min)	740	0.99	29.6	[c]
Phytosteryl esters (≙ 5% phytosterols)	Pan frying (180°C, 5 min)	291	0.6	10.9	[77]
Phytosteryl esters (≙ 5% phytosterols)	Pan frying (180°C, 10 min)	668	1.3	25.1	[77]
Dark chocolate					
Phytosteryl esters	—	68.6	—	2.9	[78]
Phytosteryl esters	Storage (5 months, 30°C)	71	0.003	3.0	[78]

a) Calculated as percentage of POPs with respect to the initial phytosterol content.
b) Calculated on the basis of consumptions corresponding to 3 g phytosterols per day.
c) http://acnfp.food.gov.uk/assess/fullapplics/phytosterol (accessed Oct 16, 2014).

oxidation rates did not reflect the additionally determined losses of initial phytosterols. For example, heating in the Schaal oven resulted in similar amounts of POPs as electric heating, both procedures leading to oxidation rates of 0.1%. At the same time, the determined loss of initial phytosterols was 4% after heating in the Schaal oven, but 60% after electric heating. This confirms the above-mentioned gap in mass balances based on the currently employed analytical procedures focusing solely on the polar POPs.

The oxidation rates determined in commercially produced, nonheated spreads enriched with phytosteryl esters [67, 73, 75] were in the same order of magnitude (0.1%) as those in nonheated milk [74, 76]. The effect of heating has been investigated in a liquid spread enriched with phytosteryl esters; treatment at 205°C for 30 min resulted in a more than tenfold higher content of POPs compared to nonheated spreads, corresponding to an oxidation rate of 1.0% (http://acnfp.food.gov.uk/assess/fullapplics/phytosterol; accessed Oct 16, 2014). This is in the same order of magnitude as oxidation rates determined in experiments investigating the effect of pan-frying at 180°C on the oxidation of sitosterol in rapeseed oil and liquid margarine enriched with phytosteryl esters [77].

The effects of storage were followed in a dark chocolate enriched with phytosteryl esters; after 5 months at 30°C, the additionally formed amount of POPs was low and corresponded to an oxidation rate of only 0.003% [78]. On the other hand, storage of a phytostanyl ester-enriched spread resulted in the highest oxidation rates reported in nonheated enriched spreads [79].

The data obtained upon storage of spread enriched with phytostanyl esters are in contrast to the view that phytostanols and their fatty acid esters are less susceptible to oxidation reactions than phytosterols and the corresponding esters due to the completely saturated ring structure [49, 60]. This assumption is supported by the tenfold lower oxidation rate observed in pasteurized milk enriched in phytostanyl fatty acid esters compared to milk enriched to the same extent with phytosteryl fatty acid esters [76]. However, intra- and intermolecular reactions, e.g. the promotion of oxidation of the stanol moieties by oxidized fatty acid moieties, may influence the formation of POPs [60]. Therefore, not only the initial phytosterol/phytostanol composition, but also the composition of the fatty acid moieties has to be taken into account when assessing the oxidative potential of an enriched food and the potentially resulting loss of functional ingredients.

The few data available demonstrate the complexity of the processes underlying the oxidation of phytosterols/phytostanols and their esters added to foods. The determined concentrations of POPs are the sums of keto-, hydroxy-, and epoxy-compounds, as shown in Fig. 1. The interpretation of the data is hampered by the fact that the employed analytical methods are not standardized; thus, the actually covered POPs may differ not only quantitatively but also qualitatively. Analytical methods allowing the investigation of intact oxidized phytosteryl and phytostanyl fatty acid esters without hydrolytic cleavage of the ester bond are at the beginning [65]. Systematic studies on the impact of the food matrix on the oxidation of phytosterols/phytostanols and their esters are lacking.

4 Estimation of dietary exposure to POPs

The available data on the occurrence of POPs and the consumption of foods enriched with phytosteryl/phytostanyl esters have been used in two approaches.

One approach is based on (i) the use of the experimentally determined contents of POPs in thermally treated enriched foods and (ii) the assumption that the upper daily intake of phytosterols/phytostanols of 3 g is achieved by consuming one of these foods. The daily intakes of POPs resulting from the consumption of the respective serving sizes corresponding to 3 g phytosterols are given for the different enriched foods in Table 2. For nonheated foods (spreads, milk, and dark chocolate), the intakes of POPs range from 1.2 to 2.9 mg/day. Upon heating, the intake is increased to 3.5–4.2 mg/day for milk and to 29.6 mg/day for liquid spread for cooking and baking.

Another approach is based on (i) the use of data on the dietary exposure to phytosterols estimated from surveys on the consumption of enriched foods [14–17] and (ii) the assumption of a minimum (0.1%) and a maximum (1%) oxidation rate. As shown in Table 3, the mean intakes of POPs resulting from the application of this approach (0.35–2.45 mg/day for a minimum and 3.5–24.5 mg/day for a maximum oxidation rate) are in a similar order of magnitude as those determined on the basis of the previously mentioned estimate.

A comparison of the estimated intakes of POPs from enriched foods (Tables 2 and 3) to those resulting from nonenriched foods (Table 1) shows significantly higher intakes to be expected from the consumption of foods with added phytosterols/phytostanols and their esters. As shown in Table 2, this increase in intake is particularly pronounced for enriched foods subjected to heating processes.

In order to estimate the intake of phytosterols from multiple sources, a worst-case model simulating prospective phytosterol intake has been developed [80], thereby assuming that the consumer does not follow the recommendations on the label [12]. Using the German National Food Consumption Study, 0.3–2 g phytosterols were hypothetically added to the usual daily servings of ten different food products selected from novel food applications; the prospective phytosterol intake was calculated by stepwise accumulation of different functional foods in three enrichment scenarios. According to the worst-case in this model, an enrichment of 2 g phytosterols per proposed serving size would result in a maximum intake of 13 g/day. Assuming again oxidation rates of 0.1 and 1%, respectively, this would result in dietary exposures to POPs of 13 mg/day and 130 mg/day, respectively.

Table 3. Intake of phytosterol oxidation products (POPs) based on consumption of enriched foods

Phytosterol intake [g/day]		POP intake [mg/day]				
		Oxidation rate 0.1%		Oxidation rate 1.0%		
Mean	95th percentile	Mean	95th percentile	Mean	95th percentile	Ref.
0.35–0.86	1.06–3.70	0.35–0.86	1.06–3.70	3.5–8.6	10.0–37.0	[14][a),b)]
0.24–0.96[d)]	1.68–2.64	0.24–0.96	1.68–2.64	2.4–9.6	16.8–26.4	[15][a),b)]
2.45	5.48	2.45	5.48	24.5	54.8	[16][c)]
1.51	4.20	1.51	4.20	15.1	42.0	[17][c)]

a) Intake calculated on the basis of purchases per household.
b) Data from The Netherlands, United Kingdom, France, Germany, and Belgium.
c) Intake calculated on the basis of purchases per consumer.
d) Data representing the median daily intake.

5 Uptake of POPs from the diet

5.1 Animal studies

Intragastric administration of two of the main classes of POPs (7-keto-derivatives and epoxides; 5 mg in 1 mL of triolein) to mesenteric duct-cannulated adult male rats revealed that the lymphatic absorption rate of 7-keto-sitosterol (1.4%) was similar to that of sitosterol (1.2%). Epoxy-derivatives showed the highest lymphatic absorption rates (e.g. α-epoxy-sitostanol: 2.7% and ß-epoxy-campestanol: 7.9%), whereby campesterol oxidation products were generally better absorbed than the respective sitosterol derivatives [81].

Administration of an AIN-93G-based diet to thoracic duct-cannulated rats (2.5 g cholesterol/kg diet or 2.5 g cholesterol/kg diet + 2.5 g phytosterols or POPs/kg diet) confirmed the low lymphatic absorption rates of phytosterols (sitosterol: 2.2%, campesterol: 5.5%) when compared to cholesterol (37.3%). However, it revealed that the lymphatic absorption rates of oxidation products of sitosterol (9.1%) and campesterol (15.9%) were actually higher than those of the parent phytosterols [82].

A mixture of POPs was fed to hamsters for 2 weeks and their concentrations were followed in plasma, aorta, liver, kidneys, and heart [83]. At the two highest doses (500 mg/kg diet and 2500 mg/kg diet), POPs were detectable in all investigated tissues. However, the proportion changed after intake: The levels of campesterol oxidation products were higher than those of the sitosterol oxidation products in plasma, while the amount of 7-keto-sitosterol, which is the dominating POP in the diet, was very low in blood. In contrast to plasma, sitostanetriol was the major POP detected in the tissues.

Similar differences of administered POPs (1 g/kg diet) were observed in a 6-week feeding study with hamsters [84]. In the employed dietary mixtures of sitosterol and stigmasterol oxidation products, the 7-keto-derivatives dominated, whereas in plasma only the 7α- and 7ß-hydroxy-derivatives and in liver 7α- and 7ß-hydroxy- as well as the 5,6α- and 5,6ß-epoxides were detected.

5.2 Human studies

The occurrence of oxidized plant sterols in human serum was first described for phytosterolemic patients [85]. Following this report, several studies reporting the presence of POPs in plasma of healthy human subjects have been published. Taken together, these studies indicate that POPs differ significantly in the amounts and type of oxidation (Table 4).

The earlier GC/MS-based studies only reported the presence of α- and ß-epoxy- and triol-derivatives [86] or the presence of 7α- and 7ß-hydroxy-derivatives [87]. The largest spectrum of POPs (in total: 11) was detected by applying GCxGC/TOF [88]. Two studies based on isotope dilution GC/MS quantified six POPs, 7-keto- and 7ß-hydroxy-sitosterol being the major representatives [89, 90]. These studies reported similar ranges of the detected POPs in two panels of 16 and 43 healthy volunteers, respectively; the determined concentration ranges of individual POPs were 0.07–3.01 ng/mL serum (0.0002–0.007 μM) [89] and 0.09–2.49 ng/mL plasma (0.0002–0.006 μM) [90].

There are only two studies available providing comparative data on the levels of POPs before and after consumption of phytosteryl ester-enriched margarine. In the first study involving 16 human subjects consuming 3 g phytosterols/day via a margarine enriched with phytosteryl esters for 28 days, there were significant increases in the serum concentrations of campesterol (from 2.82 ± 1.44 μg/mL [7.0 ± 3.6 μM] before to 4.19 ± 1.55 μg/mL [10.5 ± 3.9 μM] after the dietary intervention) and sitosterol (2.06 ± 1.27 μg/mL [5.0 ± 3.1 μM] before and 4.30 ± 1.89 μg/mL [10.4 ± 4.6 μM] after the dietary intervention) [89]. Among the detected POPs, 7ß-hydroxy-sitosterol was the major representative in the consumed margarine (8.62 ± 0.28 ng/mg). For this POP, a statistically significant increase (87%) of the serum concentration from 1.20 ± 0.54 ng/mL (0.003 ± 0.001 μM) (before consumption of the margarine) to 2.24 ± 1.25 ng/mL (0.005 ± 0.003 μM) (after consumption of the margarine) was observed. In addition, there was a highly significant correlation between the serum levels of campesterol and the sum of

Table 4. Baseline levels of phytosterol oxidation products (POPs) determined in human plasma

	POPs in human plasma/serum [μM], year [ref.]				
	2013 [90]	2012 [88]	2011 [89]	2008 [87]	2004 [86]
7α-OH-brassicasterol	—	0.0007	—	—	—
7α-OH-campesterol	0.0002	0.006	0.0002	—	—
7α-OH-stigmasterol	—	0.008	—	—	—
7α-OH-sitosterol	0.0005	0.01	0.0004	0.11	—
7ß-OH-brassicasterol	—	0.0006	—	—	—
7ß-OH-campesterol	0.0008	0.004	0.0004	—	—
7ß-OH-stigmasterol	—	0.003	—	0.11	—
7ß-OH-sitosterol	0.003	0.008	0.003	—	—
α-Epoxy-sitostanol	—	—	—	—	0.01
ß-Epoxy-sitostanol	—	—	—	—	0.13
Campestanetriol	—	—	—	—	0.01
Sitostanetriol	—	—	—	—	0.09
7-Keto-campesterol	0.001	0.002	0.001	—	—
7-Keto-stigmasterol	—	0.002	—	—	—
7-Keto-sitosterol	0.006	0.004	0.007	—	0.01
Total POPs	0.011	0.05	0.012	0.22	0.26

7-oxygenated campesterol ($R^2 = 0.915$; $p < 0.001$) and sitosterol and the sum of 7-oxygenated sitosterol ($R^2 = 0.915$; $p < 0.001$).

In a second randomized, double-blind, cross-over study, 43 healthy subjects consumed a margarine enriched with phytosteryl esters, a margarine enriched with phytostanyl esters and a control margarine, each of them for 4 weeks, separated by wash-out periods of four weeks; the consumption of the enriched margarines corresponded to intakes of 3 g/day of sterols and stanols, respectively [90]. Compared to control, the serum LDL-cholesterol concentrations were reduced after consumption of the phytosteryl ester-enriched (–8.1%) and the phytostanyl ester-enriched margarines (–7.8%). The consumption of the phytosteryl ester-enriched margarine did not result in changes of the concentrations of POPs in plasma, the individual POPs ranging from 0.09 to 2.49 ng/mL plasma (0.0002–0.006 μM) before and from 0.09 to 2.35 ng/mL plasma (0.0002–0.006 μM) after the dietary intervention. On the other hand, the intake of the phytostanyl ester-enriched margarine reduced the serum concentration of 7ß-hydroxy-campesterol by 0.07 ng/mL when compared to the control (~14%) and the phytosteryl ester-enriched margarine (~15%).

The second study revealed large variations in the baseline plasma POP concentrations among the study subjects; however, they remained relatively stable over time. Serum concentrations of (nonoxidized) sitosterol and campesterol did not correlate with plasma concentrations of sitosterol and campesterol oxidation products during any of the three interventions [91]. Six subjects could be arbitrarily classified as having consistently low or high plasma POP concentrations. This differentiation into "low and high oxidizers" was also reflected in oxidized LDL concentrations. However, oxidative and antioxidative capacity markers such as iron/copper status, α-tocopherol concentrations, and trolox equivalent antioxidant capacity values could not explain these differences.

In a recent study, the concentrations of the phytosterols campesterol and sitosterol and their oxidation products were determined in plasma and aortic valve cusps of patients with severe aortic stenosis [21]. The absolute and cholesterol corrected levels of campesterol and sitosterol in plasma, in the aortic valve cusps, and between both compartments showed a strong correlation. In contrast, the correlation between the concentrations of the phytosterols and those of the corresponding POPs in plasma and the correlation between the POP levels in plasma and those in aortic valve cusps were only weak. Moreover, the concentrations of plant sterols and those of their 7-oxidized metabolites in the tissue of aortic valve cusps significantly correlated. The authors speculated that the latter finding could relate to local inflammatory processes in atherosclerotic plaques and tissues, which generate free radicals and trigger oxidation processes.

5.3 Endogenous formation of POPs

The endogenous formation of POPs has been shown in various in vitro experiments. In rat liver mitochondria and fractions, oxidations of both the sterol nucleus and the side chain of ß-sitosterol were observed; however, the conversion rates of ß-sitosterol were far below those of cholesterol [92–95]. In rat liver mitochondria, the side chain hydroxylation of campesterol occurred at a rate similar to cholesterol [94]. In addition, as the microbial formation of cholesterol oxidation products in the gut has been observed in humans and rats, such transformation reactions are also being discussed for POPs [96, 97].The different routes of formation, i.e. enzyme-catalyzed versus chemical oxidations, are expected to be reflected in differences between the spectra of endogenously formed POPs and those ingested via the diet.

6 Biological effects of POPs

6.1 Genotoxicity

Genotoxicity was assessed in vitro using a heat-treated mixture of phytosterols containing approximately 30% POPs [98]. According to the results obtained from a bacterial mutation assay, a chromosome aberration assay and a micronucleus assay, phytosterol oxides are not considered to possess genotoxic potential. A study employing fractions isolated from thermo-oxidized ß-sitosterol confirmed that individual POPs did not show mutagenic activity toward *Salmonella typhimurium* strains [99]. No evidence of genotoxic effects in vivo was observed in a flow cytometer-based micronucleus assay in murine erythrocytes after intraperitoneal injection of mixtures of phytosterol epoxides or phytosterol triols [100].

6.2 Subchronic toxicity

A 90-day feeding study in rats was performed using a heat-treated mixture of phytosterols containing approximately 30% POPs [98]. Rats were fed a control diet without added sterols or diets with either steryl esters (5.6%) or steryl esters supplemented with 0.2, 0.6, or 1.6% of this mixture of POPs. There were no effects on behavior, food, and water consumption, ophthalmoscopy, urinalysis and renal concentrating ability, gross necropsy and histopathology. At the highest dose tested, there were significant changes when compared to the control and the diet containing only steryl esters; they comprised a slight reduction in body weight (females), slight increases in the thrombocyte count (males), and decreases in the hemoglobin level, packed cell volume, and mean corpuscular volume (females), slight decreases in the glucose level and increases in the albumin level as well as the albumin/globulin ratio (males), increases in γ-glutamyl transferase activity (females), reduced triglyceride and phospholipid levels (both sexes), and a slight increase in liver weight (females). None of these findings were supported by histopathological changes. According to these findings, a no observed effect level based on the mid dose (0.6% of the mixture of POPs in the diet) was established at an estimated dietary level of POPs of 128 mg/kg/day for males and 144 mg/kg/day for females.

6.3 Cytotoxicity and proinflammatory potential

Numerous in vitro experiments incubating various cell types and cell lines with mixtures of oxides or synthesized pure oxides showed that POPs exert cytotoxic effects, which are qualitatively similar to those observed for cholesterol oxidation products, but higher concentrations were required ($> 60\ \mu M$) [101–105]. The assessment of markers indicative of inflammatory and/or apoptotic cellular mechanisms demonstrated a reduction of cell viability as well as the generation of oxidative stress and related processes thereof, 7-ß-hydroxy- and 7-keto-derivatives exhibiting the highest cytotoxic potential among those oxides dominating in foods.

One study investigated the release of the cytokines tumor necrosis factor α (TNFα), interleukin (IL)-8, and IL-10 in the intestinal epithelial cell line Caco-2 upon addition of 7-keto-cholesterol and 7-keto-stigmasterol at a concentration of 60 μM [106]. It was shown that 7-keto-stigmasterol significantly increased the release of the three above-mentioned cytokines. Moreover, the amounts of the proinflammatory mediators TNF-α and IL-8 released upon incubation with 7-keto-stigmasterol were significantly higher than those secreted upon addition of 7-keto-cholesterol. One in vivo study investigated the effect of mixtures of sitosterol oxidation products or stigmasterol oxidation products after being injected at a concentration of 5 μM into mealworms [107]. In accordance with the in vitro observations, the administered POPs were shown to be cytotoxic, thereby inducing an increase in mealworm mortality, but their activities were five times lower than those of cholesterol oxidation products.

6.4 Pro-atherogenic effects

In vivo generated as well as dietary cholesterol oxidation products have been shown to be closely associated to atherosclerotic processes [29, 30, 33]. Regarding potential proatherogenic effects of POPs, Yang et al. [108] analyzed in vitro the effect of sitosterol and sitosterol oxidation products on the acetylcholine (ACh)-induced relaxation of rat aortae, a marker of vascular health, by isometric tension measurements. Whereas sitosterol did not impair vasorelaxation, sitosterol oxidation products significantly attenuated ACh-mediated relaxation at a concentration of 1 μg/mL (2.3 μM). This effect observed in vitro was considered by the authors to be an indicator for a proatherogenic potential of POPs and was ascribed to an increased production of reactive oxygen species.

However, data on this issue are inconclusive when considering the available in vivo experiments. An in vivo study in apolipoprotein E-deficient mice could not establish a correlation between dietary administered POPs (0.2 g/kg diet) for nine weeks and serum cholesterol concentrations as well as the size of atherosclerotic plaques when compared to a phytosterol-supplemented diet (0.2 g/kg diet) [82]. A recent study by Plat et al. [109] investigated the effects of a western-like diet containing 0.25% cholesterol compared to the same diet in which 10% of the cholesterol had been replaced by cholesterol oxidation products or dietary POPs in LDL receptor$^{+/-}$ mice for 35 weeks. Concerning the lesion size, no differences could be observed among the three groups, confirming the results obtained by the above-mentioned study. However, there was a significantly higher proportion of severe atherosclerotic lesions not only in the mice having been fed the diet containing cholesterol oxidation products but also in the group having received the POPs.

6.5 Loss of anti-atherogenic properties

Besides potential proatherogenic effects, a lower anti-atherogenic potency of phytosterols due to oxidation when compared to nonoxidized phytosterols is being discussed. Such an effect would be of particular relevance considering the functionality and efficacy of phytosterol-enriched foods.

Tomoyori et al. [82] showed that Apo-E-deficient mice had elevated cholesterol oxidation product serum levels after having been fed a diet containing POPs (0.2 g/kg diet) for nine weeks in comparison to a phytosterol-fed control group (0.2 g/kg diet). Furthermore, Liang et al. [84] fed diets containing either 1 g/kg phytosterols (sitosterol/stigmasterol) or 1 g/kg of the corresponding oxidation products to male hamsters for six weeks. The aortic plaque size and aortic cholesterol levels were reduced in the animals having received the phytosterol diets when compared to a control group; these effects were not observed upon consumption of the diets containing POPs. In addition, aortic contractions in response to ACh stimulation were significantly reduced in the phytosterol-fed group if compared to the animals fed a control diet. In turn, the aortae of the animals having consumed POPs exhibited contractions as strong as the control group, indicating that oxidation results in a loss of the cardio-protective properties of phytosterols.

6.6 Potential Effects of POPs on intestinal cholesterol transporters

The Niemann–Pick C1 like 1 protein is known to play an essential role for active uptake and absorption of cholesterol in the intestine. An inhibition of cholesterol uptake via this transporter by phytosterols/-stanols has been discussed as one of the mechanisms underlying their cholesterol lowering properties [110]. Incubating Caco-2 cells with either 7-keto-cholesterol or 7-keto-stigmasterol (60 μM) showed no effect on the expression of Niemann-Pick C1 like 1 protein compared to nontreated cells [106]. This is in agreement with data in hamsters in which neither the dietary administration of phytosterols (1 g sitosterol/stigmasterol/kg diet) nor that of the corresponding oxidation products (1 g/kg) for six weeks resulted in an altered expression of this transporter [84]. Both studies also investigated the potential impact of POPs on the active secretion of cholesterol back into the intestinal lumen, a process that is mediated by the two half transporters, ATP-binding cassette transporter G5 (ABCG5) and ATP-binding cassette transporter G8 (ABCG8) [110]. In the in vitro study, a downregulation of ABCG5 mRNA was induced by both 7-keto-stigmasterol and 7-keto-cholesterol [106]. In the in vivo study in hamsters this effect was observed not only for POPs but also for the intact phytosterols [84]. A downregulation of ABCG5 would lead to an increased intracellular cholesterol concentration and thus an enhanced availability of cholesterol for esterification and incorporation into chylomicrons; however, in both studies no effect on the expression of ABCG8 mRNA was observed. Taken together, the data on the impact of POPs on the active intestinal transport of cholesterol are still limited, and further investigation is needed.

6.7 Potential impact of POPs on the hydrolysis of phytosteryl esters

The essential precondition for the cholesterol lowering properties of phytosterols added to foods as their fatty acid esters is the intestinal cleavage of the ester bonds. In a mechanistic study Julien-David et al. [111] determined the impact of oxidation on the in vitro activity of pancreatic cholesterol esterase using sitosteryl oleate and the oxidation products 7-keto-sitosteryl oleate and sitosteryl-9,10-dihydroxystearate as substrates. As shown for 7-keto-sitosteryl oleate, the oxidation of the sterol moiety led to an increased affinity to the cholesterol esterase and a faster conversion when compared to sitosteryl oleate. In contrast, the oxidative modification of the fatty acid moiety leading to sitosteryl-9,10-dihydroxystearate resulted in an almost complete loss of hydrolysis. In addition, in the presence of sitosteryl-9,10-dihydroxystearate the hydrolysis rate of sitosteryl oleate was significantly decreased.

The observations made in the above-mentioned in vitro study illustrate the complexity of the aspects that need to be considered in the course of an assessment of POPs. Commercially, foods are almost exclusively enriched with phytosteryl/phytostanyl esters rather than with the free sterols/stanols. Owing to the analytical capabilities, the knowledge generated so far is focusing on the oxidized sterols/stanols. Alkaline hydrolysis or interesterification, which are commonly applied in the course of the analysis, results in a loss of knowledge regarding the formation of oxidized esters. The demonstrated interactions between the various oxidized "species" that may be formed during the oxidation process and the impact on the hydrolysis rate of the intact steryl ester demonstrate that it may be insufficient to focus solely on POPs when it comes to the assessment of foods enriched with various mixtures of fatty acid phytosteryl and phytostanyl esters.

7 Knowledge gaps and research needs

In summary, the review of data on POPs revealed the following knowledge gaps and research needs in order to perform a safety assessment:

(i) Analysis, occurrence and dietary exposure
   a. The presently employed analytical procedures almost exclusively focus on the polar POPs. The calculation of mass balances demonstrates a gap: losses of intact phytosterols cannot be explained by the formed amounts of these polar POPs. On the other hand, investigations of primary (hydroperoxides) and tertiary oxidation products (dimers, oligomers,

polymers) as well as oxidized fatty acid esters are still at the beginning.

b. Systematic studies on the impact of storage, heating processes (including the respective time/temperature profiles), food matrix, and presence of antioxidative compounds on the oxidation of phytosterols/phytostanols and their esters are lacking.

c. There are data on POPs in several foods containing phytosterols/phytostanols or their esters as naturally occurring constituents. However, the systematic assessment of intakes resulting from these background levels is lacking. The data available for the estimation of dietary exposure to POPs via enriched foods are limited.

(ii) Absorption and endogenous formation

a. The absorption rates of dietary POPs and their distribution to various tissues observed in animal studies differ depending on the type of phytosterol and the type of oxidation product. There are qualitative and quantitative differences between POPs administered via the diet and those determined in plasma or tissues. Predictions regarding the spectrum and the amounts of POPs upon absorption/metabolization of dietary mixtures of POPs are not possible.

b. There are significant differences in the type of oxidation products and in the concentrations regarding the reported baseline levels of POPs in humans arising from the natural occurrence of phytosterols. The data do not allow to conclude whether the observed variability is due to methodological differences or to actual exposure/biological differences. Data on the additional contribution to be expected from the consumption of enriched foods to the levels of POPs in humans are scarce.

c. There is a lack of knowledge regarding the fate of dietary POPs upon consumption of enriched foods (including the potential role of the gut microbiota) and the contributions of POPs ingested via the diet versus those endogenously formed to levels in human tissues.

d. Further studies on the presence of POPs in aortic valve tissue observed in patients with severe aortic stenosis are needed, in order to increase the understanding of a potential relationship between POPs and cardiovascular risk.

(iii) Biological effects

a. There is substantial evidence from in vitro and animal studies for reduced cholesterol-lowering properties of phytosterols upon oxidation. However, this effect has only been observed under experimental conditions involving the complete substitution of the nonoxidized phytosterol by the corresponding oxide. The data do not allow to draw conclusions regarding these effects for more realistic scenarios, i.e. consumption of foods in which the phytosterols are oxidized at a much lower rate (0.1–1%).

b. Further studies on the potential proatherogenic effects of POPs observed in in vitro and animal experiments are needed.

c. The potential inflammatory effect of POPs on intestinal epithelial cells indicated by one in vitro experiment remains unclear.

d. Knowledge on potentially adverse effects of nonidentified POPs and of oxidized phytosteryl/phytostanyl esters (either oxidized at the sterol/stanol nucleus or in the fatty acid chain) is lacking.

e. A risk-benefit analysis of the cholesterol-lowering effects of phytosterols/phytostanols and the potential adverse effects of the POPs inherently present in enriched foods is lacking.

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