DOI 10.1002/mnfr.200700025 Mol. Nutr. Food Res. 2007, *51*, 888–898

Research Article

Oxidation products of stigmasterol interfere with the action of the female sex hormone 17β-estradiol in cultured human breast and endometrium cell lines

Heike Newill[1], Renate Loske[1], Jörg Wagner[1], Christian Johannes[2], Reinhard L. Lorenz[2] and Leane Lehmann[1]

[1] Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe, Kaiserstraße, Karlsruhe, Germany
[2] Institute for the Prophylaxis of Cardiovascular Diseases, University of Munich, Munich, Germany

Phytosterols are constituents of plant membranes and are thus contained in low concentrations in vegetable products as well as at high concentrations in functional food designed to reduce serum cholesterol levels. Similar to ChOL, phytosterols are oxidized chemically in food and by biotransformation *in vivo*. Although oxyphytosterols have been detected in the serum of healthy human subjects, little is known of their biological activity. Therefore, the estrogenic and antiestrogenic activities of a mixture of six oxidation products of stigmasterol (oxy-StOL) were determined at the following endpoints: (i) the affinity to isolated human estrogen receptors (ER), (ii) the basal and 17β-estradiol (E2)-induced expression of the alkaline phosphatase (AlP) in human endometrial adenocarcinoma (Ishikawa) cells, and (iii) the basal and E2-induced proliferation of human breast adenocarcinoma (MCF-7) cells. Oxy-StOL was able to replace E2 from human ERα and ERβ and induced a weak estrogenic response in MCF-7 cells. Moreover, the E2-induced activity of the AlP in Ishikawa cells as well as the E2-induced proliferation of MCF-7 cells were decreased at noncytotoxic concentrations (up to 10 μM), indicating that at least one component of oxy-StOL represents an estrogen-active compound which might interfere with endogenous estrogens.

Keywords: Estrogen-active compounds / Ishikawa cells / MCF-7 cells / Oxyphytosterols / Stigmasterol

Received: January 23, 2007; revised: April 11, 2007; accepted: April 11, 2007

1 Introduction

Phytosterols are compounds present in all plants and in food products with plant origin (reviewed by [1, 2]). Their chemical structure is characterized by a tetracyclic cyclopenta(α)phenanthrene ring and a flexible side-chain at C_{17} [3]. Thus, the most common phytosterols, β-sitosterol, campesterol, and stigmasterol (StOL, Fig. 1) are structurally closely related to cholesterol (ChOL, Fig. 1).

The ChOL-lowering effects of phytosterols have been known for more than 50 years [4–7]. An increased intake of about 2 g of phytosterols or phytostanols *per* day lowers the ChOL absorption [8, 9] by about 10% [10–12].

Usually, cereals are the major source of phytosterols followed by margarines and vegetable oils. Levels between 350 and 1200 mg *per* kg have been observed and the dominating phytosterol is sitosterol (49–64%). The contributions from vegetables, fruits, and berries are also significant (20–25%) and the main phytosterol in these products is β-sitosterol, followed by campesterol and StOL [13]. The dietary intake of phytosterols ranges from 150 to 400 mg per day [13] and the absorption of phytosterols is below 10% [12]. During the 1990s, the esterification of sterols was reported and the possibility to include sterols in food matrices increased dramatically. The first commercial product containing esters of phytostanols was a spread called Benecol® that was launched in 1995. In some countries

Correspondence: Dr. Leane Lehmann, Institute of Applied Biosciences, Section of Food Chemistry and Toxicology, University of Karlsruhe, Kaiserstraße 12, D-76131 Karlsruhe, Germany
E-mail: leane.lehmann@lmc.uni-karlsruhe.de
Fax: +49-721-608-7255

Abbreviations: AlP, alkaline phosphatase; **ChOL,** cholesterol; **4,6-dehydro-StOL,** (24S)-ethylcholest-4,6,22-trien,3β-ol; **E2,** 17β-estradiol; **ER,** estrogen receptor; **FCS,** fetal calf serum; **HPRT,** hypoxanthine guanine phosphoribosyltransferase; **ICI 182,780,** 7-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17-diol; **oxy-StOL,** oxidation products of StOL; **StEN,** (24S)-ethylcholest-3,5,7,22-tetraen; **StOL,** stigmasterol, **TMS,** trimethylsilyl ether

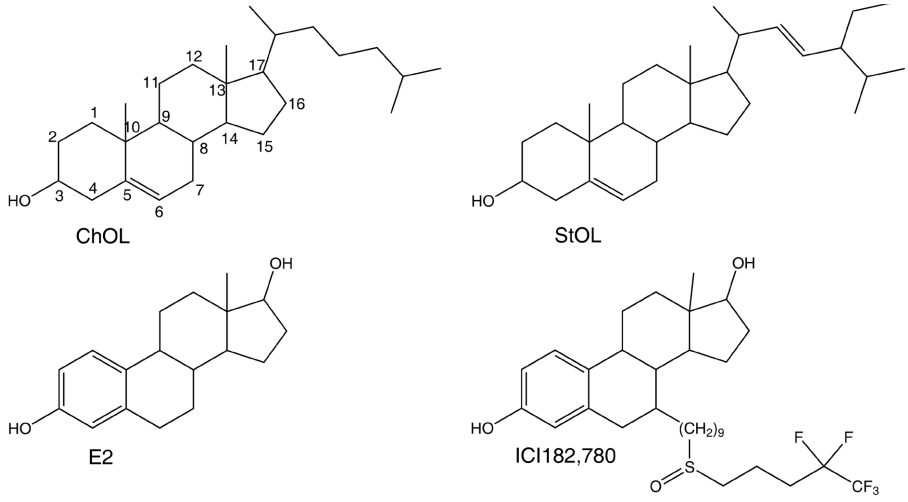

Figure 1. Chemical structure of ChOL, StOL, E2, and ICI 182,780.

(*e.g.*, Finland), there are many products enriched with phytosterols or phytostanols on the commercial market and enriched margarines have been marketed worldwide, *e.g.*, Becel® containing esters of phytosterols, was launched in 1999 in the US and in 2000 in Germany [14].

The chemical nature of phytosterols makes the molecules susceptible to oxidation. Due to similar structures (Fig. 1), much of the knowledge of the oxidation mechanisms for phytosterols refers to studies on ChOL. The oxidation of sterols is a free radical mechanism that can be initiated by factors such as heat, light, air, water, and transition metals. Sterols can be oxidized by many different oxygen species such as ground-state oxygen, ozone, singlet oxygen, hydroperoxides, dioxygen cation, and hydroxy radical (summarized by [15]). Recently, it was demonstrated that a spread fortified with phytosterols contained numerous oxidation products and oxyphytosterols were characterized with relative retention times and GC-MS. Due to poor separation no quantitative analysis was performed but a level of 68 µg oxyphytosterols *per* gram spread was estimated [16]. *In vivo* metabolism of phytosterols represents another possibility for the generation of oxyphytosterols. After administration of tritiated sitosterol to patients, normal tritiated bile acid analogues were recovered [17]. Since the first step in bile acid synthesis is hydroxylation of ChOL by cytochrom P450-dependent monooxygenases (CYPs), phytosterols seem to be substrates for ChOL-metabolizing CYP isozymes such as CYP7A or CYP27A1 involved in bile acid synthesis. Moreover, CYP11A1-catalyzed side chain cleavage which initiates the synthesis of steroid hormones has been observed after incubation of β-sitosterol with microsomes from human sterodogenic tissues [18].

The toxic effects of oxidation products of ChOL (oxy-ChOL, the term is sometimes exclusively used for derivatives of ChOL oxidized at the alkyl side chain, however it is used in a broader sense in the following paragraphs) have been extensively studied and the relation between high levels of oxy-ChOL and coronary heart diseases has been reported (summarized by [1]). Some oxy-ChOLs can be atherogenic, cytotoxic, mutagenic, cancerogenic, and may also inhibit ChOL biosynthesis and membrane functions [19]. Even though similar oxidation products can be generated from phytosterols and have been observed in animal tissue as well as in rat and human blood [20, 21], the biological effects and safety aspects of oxyphytosterols are still rather unclear [22]. Due to their sterol structure, oxyphytosterols might interfere with the action of endogenous steroid hormones such as the female sex hormone 17βestradiol (E2) and could thus exhibit hitherto unknown beneficial but also possibly adverse properties.

The present study investigates the hormonal activity of a mixture containing several oxidation products of StOL (oxy-StOL) at the endpoints (i) affinity to isolated human estrogen receptors (ERs), (ii) expression of the alkaline phosphatase (AlP) in Ishkawa cells, and (iii) proliferation of MCF-7 cells (E-screen). The oxy-StOL mixture stimulated the ER-dependent proliferation of MCF-7 cells but not the expression of AlP in Ishikawa cells. Moreover, the E2-induced proliferation of MCF-7 cells as well as the E2-induced expression of AlP in Ishikawa cells was reduced by the oxy-StOL mixture, indicating the presence of one or more endocrine-active components in this mixture.

2 Materials and methods

2.1 Chemicals

The oxy-StOL mixture was generated by means of oxidation of StOL, catalyzed by hydroxybenzotriazole/PBO_2 as described previously [23] and dissolved in ethanol (8.56 µg/µL). StOL (analytical standard, 10 mg/mL in chloroform) and E2 were obtained from Sigma (Taufkirchen, Germany). 7s-[9-[(4,4,5,5,5-Pentafluoropentyl)-sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17-diol (ICI 182,

780) was purchased from Tocris (Bristol, UK). All other chemicals, cell culture media, and medium supplements were obtained from Sigma or Roth (Karlsruhe, Germany) if not specified otherwise.

2.2 Determination of the composition of the oxy-StOL mixture

2.2.1 Derivatization

Before analysis, 2 μL of oxy-StOL mixture was dried under a stream of nitrogen and derivatized to their trimethylsilyl (TMS) ethers by reaction with *N*,*O*-Bis(TMS)trifluoroacetamide containing 1% trimethylchlorosilane.

2.2.2 GC/MS

A Finnigan GCQ capillary gas chromatograph equipped with a 30-m × 0.25-mm-id, 0.25 μm, DB-5 fused-silica column (Supelco, Bellefonte, PA) and coupled to an IT detector was operated with electron impact ionization at 70 eV (Thermo Finnigan, Austin, TX). Oven temperature was programmed from 200 (1-min hold) to 300°C (17-min hold) at a rate of 4°C/min. Temperatures of injector, transfer line, and ion source were 275, 275, and 250°C, respectively. Samples (1 μL) were injected using programmed temperature vaporization splitless injection with helium as carrier gas at a flow rate of 40 cm/s. Mass spectra were scanned from *m/z* 50 to 650 at a rate of 0.5 s/scan.

2.3 Receptor binding assay

The affinities to recombinant human ERα and ERβ were determined according to Kuiper *et al.* [24]. Briefly, 200–300 pM ERα or ERβ (Invitrogen, Karlsruhe, Germany) were mixed with 3×10^{-9} M 2,4,6,7-^{3}H-E2 (89 Ci/mmol, Amersham, Freiburg, Germany) and various concentrations of StOL (dissolved in DMSO), oxy-StOL (dissolved in ethanol) or E2 (dissolved in ethanol) in binding buffer (20 mM HEPES, 150 mM KCl, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 10% w/w glycerol, and 1 mg/mL γ-globulin) to give a total volume of 250 μL. The final concentration of solvent was 1%. The mixtures were incubated at 4°C for 20 h, then 250 μL of a cold aqueous suspension of hydroxyapatite (50% v/v, Calbiochem, Bad Soden, Germany) was added, and the mixtures were further incubated at 4°C for 15 min. Subsequently, unbound oxy-StOL and labeled and unlabeled E2 were removed by centrifugation (2500 rpm, 1 min) and by washing the pellet three times with binding buffer at 4°C. The pellet was transferred into scintillation vials (Zinsser Minis 2001, Zinsser, Frankfurt, Germany) with 2 × 100 μL of ethanol, 4 mL scintillation cocktail (Rotiszint 22, Roth, Karlsruhe, Germany) were added, and the scintillation was measured using a Beckmann LS 5000TD scintillation counter (Beckmann, Munich, Germany). EC_{50}-values were determined using the Origin® program of Microcal® Software (Northampton, MA).

2.4 Cell culture conditions

Ishikawa cells [25] were kindly provided by Dr. Ken Korach (National Institute of Environmental Health Sciences, NC). Cells were cultured in DMEM/F12 without phenol red with 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and supplemented with 10% fetal calf serum (FCS, Life Technologies, Karlsruhe, Germany). MCF-7 BUS cells [26] were kindly provided by Dr. Ana Soto (Tufts University, Boston, MA). MCF-7 cells were grown in DMEM with 4 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin supplemented with 5% heat-inactivated FCS.

2.5 Determination of the activity of AlP

The AlP assay was conducted as described previously [27]. Briefly, Ishikawa cells (2×10^4 cells *per* well) were seeded in 96-well plates (Nunc, Wiesbaden, Germany) in phenol red-free medium containing 5% charcoal/dextrane-treated FCS (Hyclone, South Logan, UT) 24 h prior to incubation with various concentrations of StOL or oxy-StOL mixture in the absence or presence of 10 nM E2 which induces maximum activity of AlP [28]. StOL, Oxy-StOL, and E2 were dissolved in DMSO and added to the medium to yield a final DMSO concentration of 0.2% v/v. Molarity of oxy-StOL solutions was based on the molecular weight of monohydroxylated StOL derivatives (428 g/M). Control experiments were carried out with medium containing 0.2% DMSO without test compounds.

After 72 h, the activity of AlP was assessed by photometric measurement of the conversion of 4-nitrophenylphosphate to 4-nitrophenol as follows: plates were washed three times with PBS-CMF pH 7.4 and lyzed at −80°C for at least 20 min. Cells were then thawed for 5 min at 20°C and 5 min at 4°C. Then, 0.05 mL 4-nitrophenylphosphate (5 mM in a buffer containing 1 M diethanolamine and 0.24 mM $MgCl_2$, pH 9.8, 4°C) were added to each well. After 5 min at room temperature, the absorption was measured at 405 nm with a microplate reader (Genios, Tecan, Crailsheim, Germany) every 10 min for 1 h.

In order to standardize the AlP activity, the number of cells *per* well of the 96-well plate was determined as follows: Ishikawa cells were grown in 96-well plates and treated with oxy-StOL and/or E2 as described above. Then, medium was aspirated, 150 μL of lysis buffer (Partec, Muenster, Germany) were added to each well, and cells were incubated at 20°C for 10 min. Nuclei were transferred quantitatively with 750 μL of staining solution (Partec) into vials (Sarstedt, Nuembrecht, Germany), and the flow cytometric determination of the cell number was carried out with a Ploidy Analyzer-II (Partec).

AlP activities were calculated as formation of 4-nitrophenol (pmol) *per* min *per* 10^5 cells.

2.6 Determination of the mRNA level of AlP

Ishikawa cells were seeded in six-well plates (6×10^5 cells *per* well) and treated with the compounds as described in Section 2.5. After 48 h, which induced maximum effect according to pilot studies, total RNA was isolated using the GenElute total RNA isolation kit (Sigma). Contaminating traces of DNA were digested with DNase (Sigma), and 1 μg of total RNA was reverse transcribed with 200 U M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) using oligo$(dT)_{18}$ primer (Fermentas) according to the instructions of the manufacturer. In addition to AlP, the expression of the housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) was determined as reference in order to compensate for variations in RNA isolation and reverse transcription as described previously [29]. The primers (Hermann GBR, Freiburg, Germany) for the cDNA of AlP were as follows: AlP forward 5′-cctaaaagggcagaag, AlP reverse 5′-gctgtagtctctgggtactca, AlP linker 5′-gctgtagtctctgggtactcacctaggatcacgtcaatc. Amplification conditions were: 94°C for 3 min; then 30 cycles: 94°C, 30 s; 53°C, 1 min; 72°C, 1 min; then 72°C for 5 min. PCR products were prestained with SYBR® Green (Life Technologies) and separated on a 2% (AlP) or 3% (HPRT) agarose gel in TAE puffer. Electrophoresis was conducted at 5 V/cm (BioRad, München, Germany) for 2 h at 4°C (AlP) or 1 h at 20°C (HPRT). PCR products were visualized after UV excitation, photographed digitally (DIANA, Raytest, Straubenhardt, Germany), and the fluorescence intensities of the PCR product of the standard (AlP, 463 bp; HPRT, 140 bp) and of the target (AlP, 505 bp; HPRT, 214 bp) were quantified using Metaview software (Molecular Devices, Sunnyvale, USA). DNA standards were generated by PCR as described by Anderson *et al.* [30]. Briefly, cDNA was amplified with the normal set of primers, the PCR product was purified by agarose gel electrophoresis, isolated by means of a DNA gel isolation kit (Amersham), and used as template in the subsequent amplification reaction which was conducted with the forward primer and the linker primer. The resulting PCR product was characterized by identical primer binding sequences as the desired sequence of the target gene, but was shorter by 42 (AlP) and 74 (HPRT) bps, respectively. The standard DNA was isolated as described before, and quantified fluorimetrically using SYBR Green.

2.7 E-screen

MCF-7 cells (2×10^4 cells *per* well) were seeded in 24-well plates (Linbro, distributed by ICN Biochemicals, Eschwege, Germany) in phenol red-free DMEM medium supplemented with 5% charcoal/dextrane-treated FCS (Hyclone) 24 h prior to incubation with various concentrations of StOL or oxy-StOL mixture in the absence or presence of 100 pM E2 which induces maximum proliferation [26]. Alternatively, cells were treated with various concentrations of E2 in the absence or presence of 10 μM oxy-StOL mixture. After 6 days, the medium was aspirated, 150 μL of lysis buffer (Partec) was added to each well, and cells were lyzed at 20°C for 5 min. Then, nuclei were stained by adding 750 μL of staining solution (Partec) and the number of nuclei was determined by flow cytometry using a Ploidy Analyzer-II (Partec).

2.8 Statistical methods

Data are expressed as mean ± SD of at least three individual experiments (new batch of cells, fresh solution of test compound), and the two-tailed independent Student's *t*-test of significant differences was conducted using the Origin program of Microcal Software.

3 Results

3.1 Composition of the oxy-StOL mixture

StOL was chemically oxidized and the oxidation products were purified as described before [23]. The components of the oxy-StOL mixture were separated by GC and detected by MS. The TIC GC/MS chromatogram of the oxy-StOL mixture demonstrated the presence of seven major compounds (Fig. 2). MS analysis revealed that at least six of those products were StOL derivatives. Based on retention time and MS data (Table 1, [16, 23, 31, 32]), four components which accounted for 17.4, 31.3, 15,9, and 4.1% of the total peak area, respectively were identified unambiguously as 7α-hydoxystigmasterol (7α-HO-STOL, (24S)-ethylcholest-5,22-dien,3β,7α-diol; Fig. 3), 7β-hydoxystigmasterol (7β-HO-StOL, (24S)-ethylcholest-5,22-dien,3β,7β-diol; Fig. 3), 7-dehydrostigmasterol (7-dehydro-StOL, (24S)-ethylcholest-5,7,22-trien,3β-ol; Fig. 3), and 7-ketostigmasterol (7-oxo-StOL, (24S)-ethylcholest-5,22-dien,3β-ol, 7-one; Fig. 3). Published retention time and MS [16, 31, 32] of the respective ChOL analogue indicated a second dehydro derivative of StOL (Table 1). Likewise 7-dehydro-StOL, it contained only one hydroxyl group (probably (24S)-ethylcholest-4,6,22-trien,3β-ol (4,6-dehydro-StOL), Fig. 3). Due to its short retention time and its MS, another component of the StOL mixture seemed to be a derivative of StOL without the hydroxyl group but containing two additional double bonds (*e.g.*, (24S)-ethylcholest-3,5,7,22-tetraen, StEN, Fig. 3). Another component of the oxy-StOL mixture (Fig. 2, peak number 3), which accounted for 3.5% of the total peak area exhibited fragment ions with *m/z* 482 (18.6), 467 (2.0), 439 (43.3), 351 (2.3), 343 (1.6), and 253 (9.0), which are characteristic for TMS derivatives of dehydro- and hydroxstigmasterols (Table 1). However, the frag-

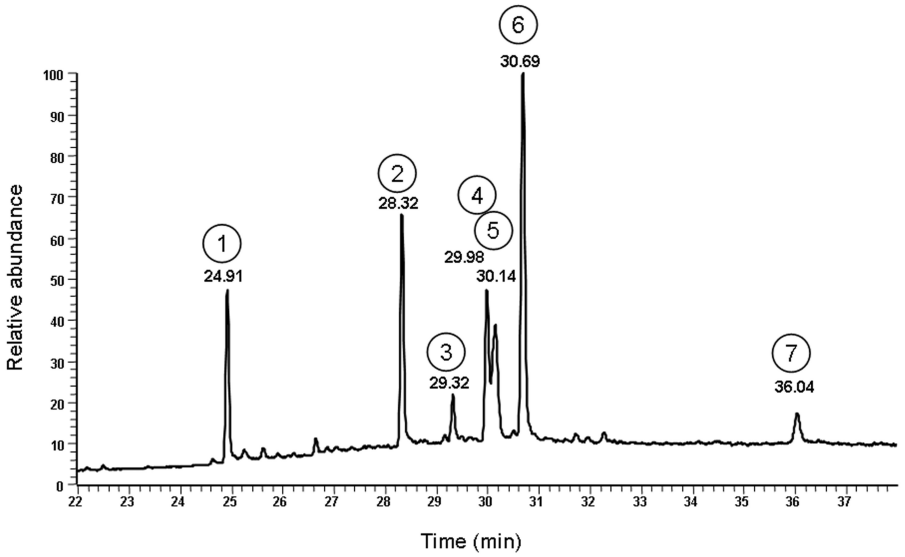

Figure 2. TIC GC/MS chromatogram of oxy-StOL. 1, possibly StEN; 2, 7α-HO-StOL; 3, unidentified compound; 4, probably 4,6-dehydro-StOL; 5, 7-dehydro-StOL; 6, 7β-HO-StOL; 7, 7-oxo-StOL.

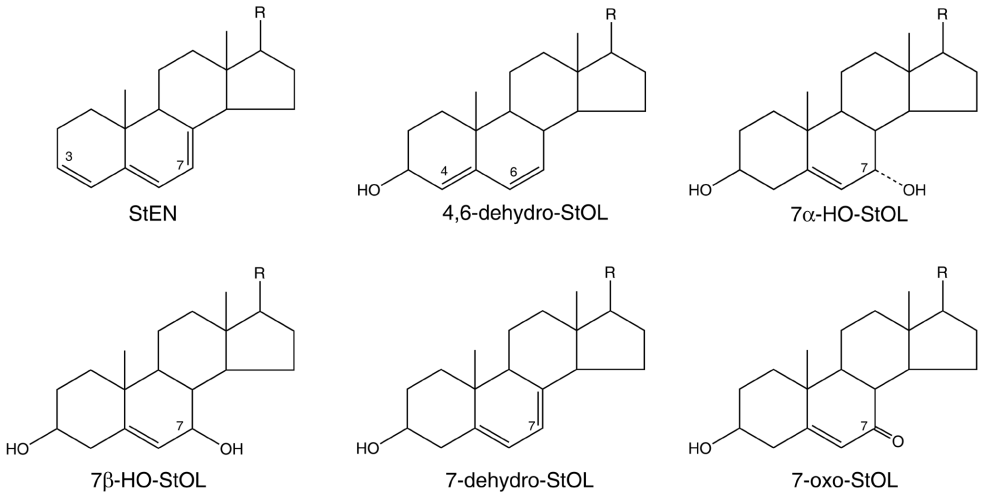

Figure 3. Chemical structures of the main components of oxy-StOL. StEN and 4,6-dehydro-StOL were not identified unambiguously. R, C_8H_{16}.

ment ions of *m/z* 438 (100), 394 (6.3), and 395 (6.1) could not be explained with the fragmentation of StOL.

3.2 Hormonal activity of the oxy-StOL mixture

3.2.1 Affinity of the oxy-StOL mixture to isolated human ERs

In order to assess the relative binding affinity of StOL and oxy-StOL to the human ERα and ERβ, the competitive displacement of ^{3}H-E2 from the recombinant receptors by these compounds was measured under cell-free conditions in comparison with E2. E2 bound with high affinity to both ERα and ERβ (Fig. 4), resulting in similar EC_{50} values (Table 2). Both StOL and oxy-StOL were also able to displace ^{3}H-E2 from human ER. However, the binding of StOL and oxy-StOL was markedly lower than that of E2 (Fig. 4 and Table 2). In comparison with E2, the affinity of StOL was about 100 000-fold lower for binding to ERα (Table 2). Due to precipitation of the test compounds before maximum displacement of ^{3}H-E2 was reached, no EC_{50} value was determined for displacement of ^{3}H-E2 from ERα by oxy-StOL and only tentative EC_{50} values were calculated for the displacement of ^{3}H-E2 from ERβ by both StOL and oxy-StOL (Table 2).

3.2.2 Influence of the oxy-StOL mixture on the ER-dependent induction of AIP in Ishikawa cells: Enzyme activity

Since binding to the ER does not necessarily confer hormonal activity, the estrogenic and antiestrogenic potential of

Table 1. Mass spectrometric data of the TMS derivatives of the main components of oxy-StOL

Compound	*m/z* (Relative abundance)					
	StEN[a)]	7α-HO-StOL	4,6-dehydro-StOL[a)]	7-dehydro-StOL	7β-HO-StOL	7-oxo-StOL
Retention time	24.91	28.32	29.98	30.14	30.69	36.04
Peak area (% total area)	12.3	17.4	12.7	15.9	31.3	4.1
M	**392 (100)**	572 (2.8)	**482 (100)**	**482 (68.3)**	**572 (9.5)**	**498 (100)**
M – CH_3	377 (4.6)		**467 (9.8)**	**467 (6.3)**	557 (1.0)	**483 (8.8)**
M – C_3H_7						**455 (18.5)**
M – TMSOH		**482 (100)**	392 (2.6)	**392 (76.3)**	**482 (100)**	**408 (19.7)**
M – (C_8H_{15} + H)						**386 (53.22)**
M – (TMSOH + CH_3)		467 (2.3)	377 (1.0)	**377 (100)**	467 (4.3)	**393 (12.7)**
M – (C_1,C_3-TMSOH + 2 H)				**351 (48.6)**	441 (1.3)	367 (3.6)
M – (TMSOH + C_3H_7)						**365 (9.1)**
M – side chain	**253 (14.6)**		343 (1.5)	**343 (6.7)**	433 (1.1)	**359 (15.2)**
M – 2 TMSOH					392 (2.4)	
M – (side chain + TMSOH)			253 (2.1)	**253 (28.5)**	343 (2.8)	**269 (23.4)**
M – (side chain + 2 TMSOH)					253 (4.4)	
C_1,C_3-TMSO				129 (4.8)	129 (2.3)	
?				**131 (9.7)**		131 (1.6)

Bold, fragment ions with a relative abundance > 5.
a) Molecule ion based on proposed structure.

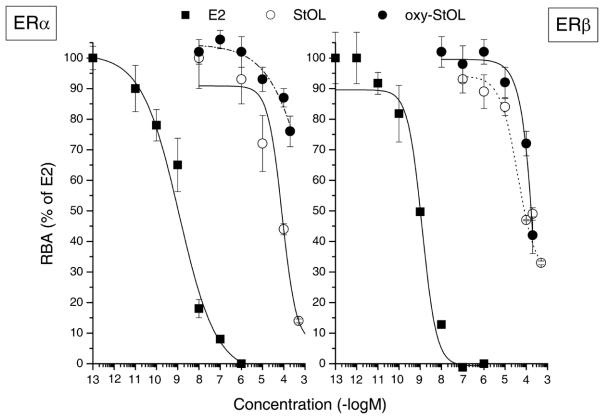

Figure 4. Competitive replacement of ^{3}H-E2 from recombinant human ERα (left) and ERβ (right) by StOL, oxy-StOL, and E2. Data represent mean ± SD of 3 – 4 independent experiments.

StOL and oxy-StOL was studied in cultured Ishikawa cells. These human endometrial cells are known to express predominantly ERα [25]. When the mRNA of both ERs were determined by RT-PCR, the mRNA of ERβ was also detected and accounted for about one tenth of the mRNA of ERα as determined by competitive PCR (Lehmann, unpublished work). AlP enzyme activity in Ishikawa cells is specifically stimulated by estrogens whereas antiestrogens such as ICI 182,780 (Fig. 1) completely block the induction of AlP activity by estradiol [25, 27]. E2 induces the activity of the AlP with an EC_{50} value of about 200 pM and maximum induction of AlP is reached with 10 nM E2 in this test system [29].

Table 2. Affinity of StOL and oxy-StOL to isolate recombinant human ERα and ERβ

	ERα		ERβ	
	EC_{50}	RBA (%)	EC_{50}	RBA (%)
E2	1.14 ± 0.40 nM	100	1.22 ± 0.04 nM	100
StOL	80 ± 10 μM	0.001	(40 ± 5.9 μM)	(0.003)
Oxy-StOL	n.d.	n.d.	(1.4 ± 23 μM)	(0.09)

RBA, relative binding affinity. Data represent mean ± SD of three independent experiments; n.d., not determined; (), tentative values since the replacement of ^{3}H-E2 was not complete.

For the assessment of the estrogenic potential of StOL and oxy-StOL, Ishikawa cells were treated with various concentrations of StOL or oxy-StOL mixture and the activity of AlP was determined after 72 h in comparison with 10 nM E2, which caused a maximum induction of AlP [28, 29]. Both StOL (data not shown) and oxy-StOL (Fig. 5) had no influence on the basal AlP activity (27.5 ± 1.2 pmol 4-nitrophenol *per* min *per* 10^5 cells).

In order to assess the antiestrogenic potential of StOL and oxy-StOL, their effect on the induction of AlP by E2 was studied (Fig. 5). Whereas up to 10 μM StOL did not influence the E2-induced AlP activity (133.1 ± 12.4 pmol 4-nitrophenol *per* min *per* 10^5 cells, data not shown), 5 and 10 μM oxy-StOL significantly reduced the E2-induced AlP activity by 18.0 ± 2.79% and 28.5 ± 2.37%, respectively (Fig. 5). The known ER-antagonist ICI182,780 reduced the E2-induced AlP activity at about 1000-fold lower concentrations than oxy-StOL (Fig.5).

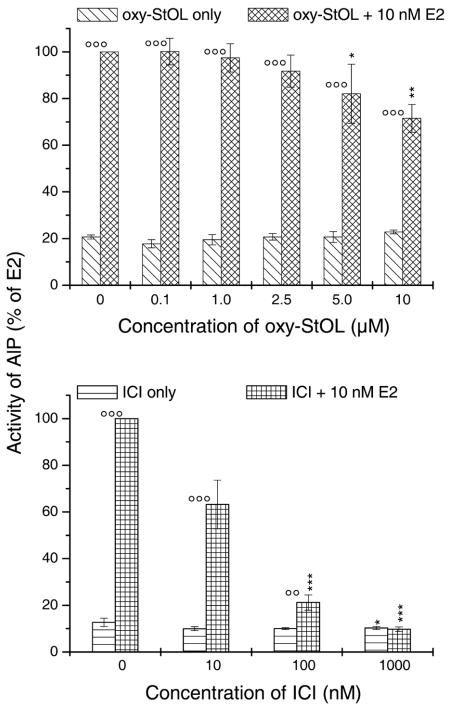

Figure 5. Relative activity of the AIP after incubation of cultured Ishikawa cells with 10 nM E2 in the presence of oxy-StOL (top panel) or ICI 182,780 (bottom panel) for 72 h. Data represent mean ± SD of four independent experiments including six wells each. Statistical significance was determined using Student's *t*-test. Asterisks indicate statistical difference of treatment with respective concentration compared to concentration "0" (DMSO control); circles indicate statistical difference between treatment groups (striped and crossed columns). Levels of significance: *, 0.05; **/°°, 0.01; ***/°°°, 0.001.

3.2.3 Influence of the oxy-StOL mixture on the ER-dependent induction of AIP in Ishikawa cells: mRNA levels

In order to verify the effect of oxy-StOL on the E2-induced expression of AlP, mRNA levels of AlP were determined after treatment of Ishikawa cells with 10 nM E2 in the absence and presence of 5 and 10 µM oxy-StOL. In the absence of StOL, treatment with 10 nM E2 increased the ratio of AlP/HPRT mRNA 34-fold compared to those of Ishikawa cells treated with solvent alone (data not shown). In contrast, in the presence of 5 µM (10 µM) oxy-StOL, the ratio of AlP/HPRT mRNA was merely increased 16-fold (11-fold) by 10 nM E2, representing a reduction to 50 (31)% (Fig. 6).

3.2.4 Influence of the oxy-StOL mixture on the ER-dependent proliferation of MCF-7 cells (E-screen)

In order to further investigate the putative antiestrogenic effect of oxy-StOL in another cell line and at another end-

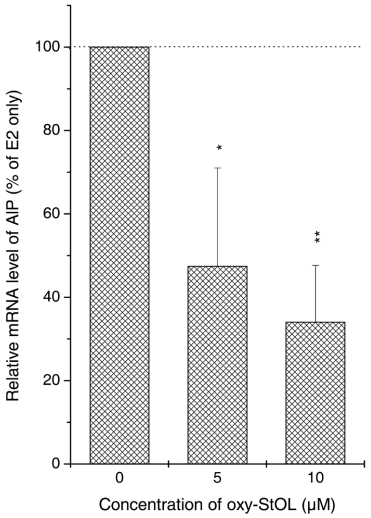

Figure 6. Expression of AIP mRNA in relation to HPRT mRNA after incubation of cultured Ishikawa cells with oxy-StOL in the presence of 10 nM E2 for 48 h relative to expression of control cells treated with 10 nM E2 only (100%). Data represent mean + SD of three PCR reactions. Statistical significance was determined using Student's *t*-test. Levels of significance: *, 0.05; **, 0.01. Results were confirmed independently in a second experiment with the same outcome.

point, the influence of oxy-StOL on the ER-mediated proliferation of MCF-7 cells (E-screen) was determined. As expected, 1 – 100 pM E2 stimulated the proliferation of MCF-7 cells in a concentration dependent-way (Fig. 7). Treatment with 1 – 100 nM E2 did not further increase the proliferation of MCF-7 cells (data not shown), indicating that maximum stimulation of proliferation was obtained with 100 pM E2. Likewise, 10 µM oxy-StOL increased the proliferation of MCF-7 cells (1.6-fold, Fig. 7), however not reaching the same maximum induction as obtained with 100 pM E2 (4.1 – 5.1-fold). When MCF-7 cells were treated with different concentrations of E2 in the presence of 10 µM oxy-StOL, the E2-induced proliferation was significantly inhibited by up to 50.5% (Fig. 7). Likewise, when MCF-7 cells were treated with 2.5 – 10 µM oxy-StOL in the presence of 100 pM E2, E2-induced proliferation (5.1-fold) was significantly inhibited by 5 µM and more oxy-StOL (Fig. 7).

In contrast, up to 10 µM StOL affected neither the basal nor the E2-induced proliferation of MCF-7 cells (data not shown). The known ER-antagonist ICI 182 780 reduced both the basal and the E2-induced proliferation of MCF-7 cells, reaching nearly complete inhibition of proliferation already at 10 nM (Fig. 7).

4 Discussion

Phytosterol-rich food such as certain plant oils and phytosterol-enriched functional food items are currently gaining

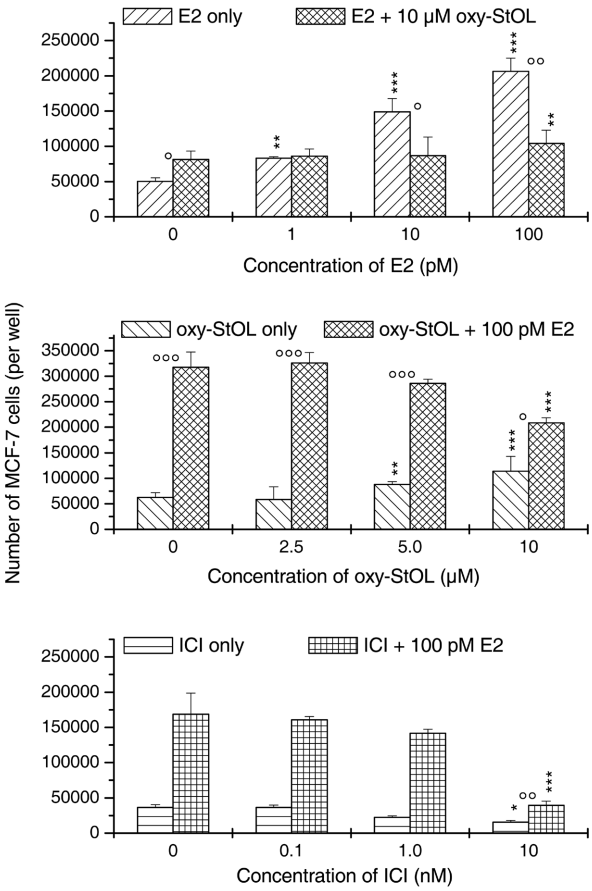

Figure 7. Influence of oxy-StOL and ICI 182,780 (ICI) on the basal and E2-induced proliferation of MCF-7 cells (E-screen). Cells were treated either with 10 µM oxy-StOL in the absence and presence of various concentrations of E2 (top panel) or with various concentrations of oxy-StOL (middle panel) or ICI (bottom panel) in the absence and presence of 100 pM E2. Data represent mean + SD of four (top panel) or three (middle and lower panel) independent experiments. Statistical significance was determined using Student's *t*-test. Asterisks indicate statistical difference of treatment with respective concentration compared to concentration 0 (DMSO control); circles indicate statistical difference between treatment groups (striped and crossed columns). Levels of significance:°, 0.05; **/°°, 0.01; ***/°°°, 0.001.

much popularity due to their putative beneficial health effects. In particular, intake of 2 g of phytosterols *per* day *via* vegetable fat supplemented with phytosterol esters is known to reduce serum ChOL levels and is thus believed to protect against cardiovascular diseases (summarized in [12]).

The present study represents the first *in vitro* study on the estrogenic activity of pure StOL and of StOL oxidation products. Our study demonstrates the modulation of the action of the hormone E2 by an oxy-StOL mixture at different endpoints in a cell-free test system as well as in two cultured different human cell lines.

The graphs of the displacement of ^{3}H-E2 from human ERs clearly demonstrate a weak affinity of StOL and oxy-StOL to human ER (Fig. 4) although EC_{50} values could not be determined for oxy-StOL due to its limited solubility. However, the qualitative result of the present study might satisfy the requirements for the characterization of the binding affinity of a mixture of various compounds. Exact EC_{50} values for the single components of the mixture will be determined in subsequent studies. In concordance with previous studies of our laboratory in the Ishikawa cell line [27, 28], more pronounced effects were observed at the AlP mRNA level than at the AlP activity level in this study. As a matter of fact, antiestrogenic effects are not easy to distinguish from cytoxicity [29]. In our case however, cytotoxicity can be excluded as reason for the observed reduction of E2-induced AlP activity in Ishikawa cells as well as the reduction of E2-induced proliferation in MCF-7 cells because (i) the AlP activity was standardized to the respective cell number and AlP mRNA levels were standardized to the housekeeping gene HPRT, (ii) the basal activity of AlP was not influenced by oxy-StOL, and (iii) the basal proliferation of MCF-7 cells was even increased by treatment with oxy-StOL.

A weak estrogenic potential of oxy-StOL was observed in MCF-7 cells but not in Ishikawa cells. The Ishikawa test system might be not sensitive enough for the detection of a very weak estrogenic response. This is supported by the fact that at higher concentrations up to 50 µM also an induction of AlP activity by oxy-StOL was observed in Ishikawa cells. However, the quantification was impaired by reduced cell numbers due to cytotoxicity (data not shown). Since the oxy-StOL mixture contains several oxy-StOL, it could not be determined whether the cytotoxic agent was identical with the hormonally active compound(s).

The present study suggests that oxy-StOL interferes with the classical ER pathway by binding to ERs. The estrogenic/antiestrogenic potential of oxy-StOL observed in the present study could have two reasons: (i) the presence of one or more ER antagonists as well as of one or more ER agonists or (ii) the presence of one or more partial ER agonists (*i.e.*, compounds which bind to the ER but do not cause the complete response) in the oxy-StOL mixture. Further studies with the isolated purified compounds present in the oxy-StOL mixture are currently under way in order to elucidate the molecular mechanism of oxy-StOL in human estrogen-responsive cells.

To our knowledge, there is only one *in vitro* study investigating the estrogenic activity of a phytosterol mixture containing StOL [33]: up to 100 µM of a phytosterol mixture (23.3% StOL, 47.9% β-sitosterol, and 28.8% campesterol) did not replace ^{3}H-E2 from ER in rat uterus cytosol. The highest concentration tested in the cited study was lower in StOL content (about 23 µM) than in the present study. Yet in the present study, significant displacement of ^{3}H-E2 from human ERα was observed already with 10 µM StOL.

StOL might have a higher affinity to human ERs than to rat ER. Furthermore, the displacement curves gained with unlabeled E2 presented in [34] exhibited an atypical linear form in a broad concentration range. Despite the affinity of StOL to isolated human ERs, no estrogenic activity of StOL was observed in two different cultured mammalian cell lines at different endpoints in the concentration range used in the present study (up to 20 μM). Likewise, the phytosterol mixture mentioned above did not stimulate the ER-dependent expression of a reporter gene in an artificial yeast system [33]. However, despite the phytosterol mixture tested contained more than 40% β-stigmasterol (which was shown to increase the proliferation of MCF-7 cells *in vitro* at 1 μM [34]) as well, no estrogenic effect was observed in the yeast system [33]. Whereas β-sitosterol increased the proliferation of MCF-7 cells *in vitro*, dietary β-sitosterol did not stimulate MCF-7 tumor growth *in vivo*. In contrast, dietary β-sitosterol reduced E2-induced MCF-7 tumor growth [34]. Similarly, the present study observed a weak stimulation of basal proliferation of MCF-7 cells *in vitro*, yet a pronounced reduction of the E2-induced proliferation of MCF-7 cells by the oxy-StOL mixture.

In contrast to the limited published data of the estrogenic activity of oxy-StOL or pure StOL, there are some studies on the hormonal activity of β-stigmasterol and phytosterol mixtures. *In vivo*, a variety of effects on the reproductive system such as antiandrogenic action in rabbits and decrease in testicular weight and sperm concentrations in rats have been reported for β-stigmasterol and phytosterol-rich extracts [35–38]. Orally administered β-sitosterol increased uterine weight in rats receiving 6.2 μg/dL drinking water for 30 days. In contrast, dietary application of a mixture of phytosterols (5–500 g/kg body weight) to immature female rats did not increase uterine weight [33] and in a two-generation reproduction study in rats, phytosterol esters had no effect on the reproduction of the F0 and F1-generations [39, 40]. Despite β-sitosterol may be converted to steroid-hormones although at a slower conversion rate than that of ChOL [18], dietary phytosterols (5.8 g/day) did not influence sex hormone levels in a 3-wk study with 12 men and 12 women (cited in [41]).

Yet, the published studies did not take into account the consumption of oxidation products of phytosterols which modulated the action of E2 in our cellular test systems. Some oxidation products detected in the oxy-StOL mixture would be formed during storage and processing of food rather than be generated metabolically *in vivo*.

Most of the peak area (93.8%) of the TIC GC/MS chromatogram of the tested oxy-StOL mixture was unambiguously identified as StOL oxidation products and another 2.7% was possibly due to minor derivatives of StOL. The structure of 4 oxidation products was identified unambiguously (7α-HO-StOL, 7β-HO-StOL, 7-dehydro-StOL, and 7-oxo-StOL). Moreover, another oxidation product was probably 4,6-dehydro-StOL and a possible structure of a second (StEN) was proposed. However, the presence of a nonsteroidal contamination, which accounted for 3.5% of total peak area could not be completely excluded. Therefore, it cannot be excluded that a non-StOL derivative contributed to the observed antiestrogenic properties of the mixture.

The thermal oxidation in the ring structure of phytosterols has been studied in detail, *e.g.*, [1, 31, 42–45]. All studies reported the formation of 7α-hydroxy-, 7β-hydroxy-, and 7-oxo-phytosterols as well as of 5α,6α-epoxides and 5β,6β-epoxides. Further oxidation of these oxyphytosterols leads to dehydration and subsequent abstraction of the hydroxy group at C_3, which generates conjugated dienes and trienes [46, 47]. This observation is in concordance with the hypothesized structures of the oxy-StOL derivatives at retention time 29.98 and 24.91 min (Fig. 2 and Table 1).

With the exception of epoxides, the oxy-StOL mixture contained thus the same oxidation products that have been observed in food and biological samples. Moreover, 7α-HO-StOL and 7-oxo-StOL might also be generated metabolically *in vivo* [17, 48]; however, much slower than the respective ChOL derivatives [17, 18].

Numerous oxyphytosterols were identified in phytosterol ester-enriched spreads and the level of oxyphytosterols were calculated to range from 12 up to 68 μg/g spread [16]. The intake of oxyphytosterols when consuming the recommended level of phytosterols-enriched spread was estimated to be up to 1700 μg/day/person [16]. StOL can be expected to account for 6–30% of the total phytosterol content of a phytosterol-enriched spread [41].

Levels of oxy-phytosterols in plasma from healthy humans ranged from around 5 ng/mL for sitostanol-5α,6α-epoxide, campestanetriol and 7-ketositosterol to a much higher level for sitostanol-5β,6β-epoxide and sitostanetriol. It was suggested that oxyphytosterols derived from either food or oxidation in the plasma [20]. In rats and hamsters, fractional resorption rates of dietary oxy-phytosterols were higher than for native phytosterols [49, 50].

Many studies have reported ChOL oxidation products to be mutagenic, cytotoxic or atherogenic (summarized by [19, 51]), while data concerning biological effects of oxyphytosterols are limited [22]. Despite the lack of hormonal activity of nonoxidized phytosterol mixtures, the relevance of phytosterol oxidation before and after consumption still needs to be clarified. The present study demonstrates that phytosterol oxidation products modulate the action of E2 *in vitro* and therefore might also interfere with steroid hormones *in vivo*. Whether this modulation would represent a hitherto unknown adverse or an additional beneficial property of dietary phytosterols still remains to be elucidated.

The authors thank Carolin Müller for helping with the AlP assay as well as with the E-screen and Doris Honig for helping with the GC/MS analysis. We are also indebted to Doris Wedlich (Institute of Zoology, University of Karls-

ruhe) for the possibility of data analysis in her laboratory. Supported by DFG (Me574/24-2).

5 References

[1] Guardiola, F., Dutta, P., Codony, R., Savage, G. (Eds.), *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence, and Biological Effects*, AOCS press, Champain 2002.

[2] Moreau, R. A., Whitaker, B. D., Hicks, K. B., Phytosterols, phytostanols, and their conjugates in foods: Structural diversity, quantitative analysis, and health-promoting uses, *Prog. Lipid Res.* 2002, *41*, 457 – 500.

[3] Piironen, V., Lindsay, D., Miettinen, T., Toivo, J., Lampi, A., Plant sterols: Biosynthesis, biological function, and their importance to human nutrition, *J. Sci. Food Agric.* 2000, *80*, 939 – 969.

[4] Dempsey, M. E., Farquhar, J. W., Smith, R. E., The effect of beta sitosterol on the serum lipids of young men with arteriosclerotic heart disease, *Circulation* 1956, *14*, 77 – 82.

[5] Peterson, D. W., Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks, *Proc. Soc. Exp. Biol. Med.* 1951, *78*, 143 – 147.

[6] Pollak, O. J., Reduction of blood cholesterol in man, *Circulation* 1953, *7*, 702 – 706.

[7] Pollak, O. J., Successive prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit, *Circulation* 1953, *7*, 696 – 701.

[8] Miettinen, T. A., Vuoristo, M., Nissinen, M., Jarvinen, H. J., Gylling, H., Serum, biliary, and fecal cholesterol and plant sterols in colectomized patients before and during consumption of stanol ester margarine, *Am. J. Clin. Nutr.* 2000, *71*, 1095 – 1102.

[9] Plat, J., Mensink, R. P., Vegetable oil based versus wood based stanol ester mixtures: Effects on serum lipids and hemostatic factors in nonhypercholesterolemic subjects, *Atherosclerosis* 2000, *148*, 101 – 112.

[10] Gylling, H., Rajaratnam, R. A., Vartiainen, E., Puska, P., Miettinen, T. A., Changes in serum level and metabolism of cholesterol with plant stanol esters in postmenopausal women with and without coronary artery disease, *Menopause* 2006, *13*, 286 – 293.

[11] Neil, H. A., Meijer, G. W., Roe, L. S., Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread, *Atherosclerosis* 2001, *156*, 329 – 337.

[12] Ostlund, R. E., Jr., Phytosterols in human nutrition, *Annu. Rev. Nutr.* 2002, *22*, 533 – 549.

[13] Piironen, V., Lampi, A., in: Dutta, P. (Ed.), *Phytosterols as Functional Food Component and Nutraceuticals*, Marcel Dekker Inc., New York 2004, pp. 1 – 32.

[14] Moreau, R. A., in: Dutta, P. (Ed.), *Phytosterols as Functional Food Component and Nutraceuticals*, Marcel Dekker Inc., New York 2004, pp. 1 – 32.

[15] Smith, L. L., Cholesterol autoxidation 1981 – 1986, *Chem. Phys. Lipids* 1987, *44*, 87 – 125.

[16] Grandgirard, A., Martine, L., Joffre, C., Juaneda, P., Berdeaux, O., Gas chromatographic separation and mass spectrometric identification of mixtures of oxyphytosterol and oxycholesterol derivatives application to a phytosterol-enriched food, *J. Chromatogr. A* 2004, *1040*, 239 – 250.

[17] Salen, G., Ahrens, E., Grundy, S., Metabolismus of β-sitosterol in man, *J. Clin. Invest.* 1970, *49*, 952 – 967.

[18] Aringer, L., Eneroth, P., Nordstrom, L., Side-chain cleavage of 4-cholesten-3-one, 5-cholesten-3 alpha-ol, beta-sitosterol, and related steroids in endocrine tissues from rat and man, *J. Steroid Biochem.* 1979, *11*, 1271 – 1285.

[19] Smith, L. L., Johnson, B. H., Biological activities of oxysterols, *Free Radic. Biol. Med.* 1989, *7*, 285 – 332.

[20] Grandgirard, A., Martine, L., Demaison, L., Cordelet, C., *et al.*, Oxyphytosterols are present in plasma of healthy human subjects, *Br. J. Nutr.* 2004, *91*, 101 – 106.

[21] Plat, J., Brzezinka, H., Lutjohann, D., Mensink, R. P., von Bergmann, K., Oxidized plant sterols in human serum and lipid infusions as measured by combined gas-liquid chromatography-mass spectrometry, *J. Lipid Res.* 2001, *42*, 2030 – 2038.

[22] Oehlr, L., Dean, L., Boyd, L., in: Dutta, P. (Ed.), *Phytosterols as Functional Food Component and Nutraceuticals*, Marcel Dekker Inc., New York 2004, pp. 1 – 32.

[23] Johannes, C., Lorenz, R. L., Preparation and mass spectrometry of 14 pure and 18O(2)-labeled oxidation products from the phytosterols beta-sitosterol and stigmasterol, *Anal. Biochem.* 2004, *325*, 107 – 116.

[24] Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., *et al.*, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, *Endocrinology* 1998, *139*, 4252 – 4263.

[25] Nishida, M., The Ishikawa cells from birth to the present, *Hum. Cell* 2002, *15*, 104 – 117.

[26] Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., *et al.*, The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants, *Environ. Health Perspect.* 1995, *103*, 113 – 122.

[27] Lehmann, L., Wagner, J., Metzler, M., Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells, *Food Chem. Toxicol.* 2006, *44*, 398 – 408.

[28] Wagner, J., Lehmann, L., Estrogens modulate the gene expression of Wnt-7a in cultured endometrial adenocarcinoma cells, *Mol. Nutr. Food Res.* 2006, *50*, 368 – 372.

[29] Lehmann, L., Esch, H. L., Wagner, J., Rohnstock, L., Metzler, M., Estrogenic and genotoxic potential of equol and two hydroxylated metabolites of Daidzein in cultured human Ishikawa cells, *Toxicol. Lett.* 2005, *158*, 72 – 86.

[30] Anderson, K. M., Cheung, P. H., Kelly, M. D., Rapid generation of homologous internal standards and evaluation of data for quantitation of messenger RNA by competitive polymerase chain reaction, *J. Pharmacol. Toxicol. Methods* 1997, *38*, 133 – 140.

[31] Bortolomeazzi, R., De Zan, M., Pizzale, L., Conte, L. S., Mass spectrometry characterization of the 5alpha-, 7alpha-, and 7beta-hydroxy derivatives of beta-sitosterol, campesterol, stigmasterol, and brassicasterol, *J. Agric. Food Chem.* 1999, *47*, 3069 – 3074.

[32] Smith, F., Korn, E., 7-Dehydrostigmasterol and ergosterol: The major sterols of an amoeba, *J. Lipid Res.* 1968, *9*, 405 – 408.

[33] Baker, V. A., Hepburn, P. A., Kennedy, S. J., Jones, P. A., *et al.*, Safety evaluation of phytosterol esters. Part 1. Assessment of oestrogenicity using a combination of in vivo and in vitro assays, *Food Chem. Toxicol.* 1999, *37*, 13 – 22.

[34] Ju, Y. H., Clausen, L. M., Allred, K. F., Almada, A. L., Helferich, W. G., beta-sitosterol, beta-sitosterol glucoside, and a mixture of beta-sitosterol and beta-sitosterol glucoside modu-

late the growth of estrogen-responsive breast cancer cells in vitro and in ovariectomized athymic mice, *J. Nutr.* 2004, *134*, 1145 – 1151.

[35] Ghannudi, S., Shareha, A., Sammannoudy, F., Adverse effects of phytoestrogens. I. Histological and histochemical effects of beta-sitosterol on the immature male rabbit, *Libyan J. Sci.* 1978, *8B*, 17 – 24.

[36] Malini, T., Vanithakumari, G., Antifertility effects of beta-sitosterol in male albino rats, *J. Ethnopharmacol.* 1991, *35*, 149 – 153.

[37] Pakrashi, A., Basak, B., Abortifacient effect of steroids from Ananas comosus and their analogues on mice, *J. Reprod. Fertil.* 1976, *46*, 461 – 462.

[38] Register, B., Bethel, M. A., Thompson, N., Walmer, D., *et al.*, The effect of neonatal exposure to diethylstilbestrol, coumestrol, and beta-sitosterol on pituitary responsiveness and sexually dimorphic nucleus volume in the castrated adult rat, *Proc. Soc. Exp. Biol. Med.* 1995, *208*, 72 – 77.

[39] Waalkens-Berendsen, D. H., Wolterbeek, A. P., Wijnands, M. V., Richold, M., Hepburn, P. A., Safety evaluation of phytosterol esters. Part 3. Two-generation reproduction study in rats with phytosterol esters – a novel functional food, *Food Chem. Toxicol.* 1999, *37*, 683 – 696.

[40] Whittaker, M. H., Frankos, V. H., Wolterbeek, A. P., Waalkens-Berendsen, D. H., Two-generation reproductive toxicity study of plant stanol esters in rats, *Regul. Toxicol. Pharmacol.* 1999, *29*, 196 – 204.

[41] SCF, *Opinion of the Scientific Committee on Food on a request for the safety assessment of the use of phytosterol esters in yellow fat spreads*, European Commission 2000.

[42] Bortolomeazzi, R., Cordaro, F., Pizzale, L., Conte, L. S., Presence of phytosterol oxides in crude vegetable oils and their fate during refining, *J. Agric. Food Chem.* 2003, *51*, 2394 – 2401.

[43] Dutta, P., Study on phytosterol oxides II. Content in some vegetable oils and in french fries prepared in these oils, *J. Am. Oil Chem. Soc.* 1997, *74*, 659 – 666.

[44] Dutta, P., LA, A., Study on phytosterol oxides I. Effect of storage on the content in potato chips prepared in different vegetable oils, *J. Am. Oil Chem. Soc.* 1997, *74*, 647 – 657.

[45] Lampi, A. M., Juntunen, L., Toivo, J., Piironen, V., Determination of thermo-oxidation products of plant sterols, *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* 2002, *777*, 83 – 92.

[46] Dutta, P., Przybylski, R., Appelqvist, L., in: Perkins, E., Erickson, M. (Eds.), *Deep Frying: Chemistry, Nutrition and Practical Applications*, AOCS Press, Champaign 1996, pp. 112 – 150.

[47] Lercker, G., Rodriguez-Estrada, M., in: Guardiola, F., Dutta, P., Codony, R., Savage, G. (Eds.), *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence and Biological Effects*, AOCS Press, Champaign 2002.

[48] Aringer, L., Eneroth, P., Studies on the formation of C7-oxygenated cholesterol and beta-sitosterol metabolites in cell-free preparations of rat liver, *J. Lipid Res.* 1973, *14*, 563 – 572.

[49] Grandgirard, A., Demaison-Meloche, J., Cordelet, C., Demaison, L., Incorporation of oxyphytosterols in tissues of hamster, *Reprod. Nutr. Dev.* 2004, *44*, 599 – 608.

[50] Tomoyori, H., Kawata, Y., Higuchi, T., Ichi, I., *et al.*, Phytosterol oxidation products are absorbed in the intestinal lymphatics in rats but do not accelerate atherosclerosis in apolipoprotein E-deficient mice, *J. Nutr.* 2004, *134*, 1690 – 1696.

[51] Osada, K., in: Guardiola, F., Dutta, P., Codony, R., Savage, G. (Eds.), *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurence, and Biological Effects*, AOCS Press, Champaign 2002, pp. 278 – 318.

