

# Endocrine Active UV Filters: Developmental Toxicity and Exposure Through Breast Milk

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**Abstract:** Several UV filters exhibit endocrine activity. Evidence for transdermal passage and presence in the food chain (fish) suggests potential exposure of humans during development. Developmental toxicity was studied in rats for the estrogenic UV filters 4-methylbenzylidene camphor (4-MBC, 0.7, 7, 24, 47 mg/kg/day) and 3-benzylidene camphor (3-BC, 0.07, 0.24, 0.7, 2.4, 7 mg/kg/day) administered in chow to the parent generation before mating, during pregnancy and lactation, and to the offspring until adulthood. Neonates exhibited enhanced prostate growth after 4-MBC and altered uterine gene expression after both filters. 4-MBC and 3-BC delayed male puberty and affected reproductive organ weights of adult offspring. Interactions with the thyroid were noted. Expression and estrogen sensitivity of target genes and nuclear receptor coregulators were altered at mRNA and protein levels in adult uterus, prostate and brain. Female sexual behavior was affected by 4-MBC and 3-BC, estrous cycles by 3-BC. Classical endpoints exhibited LOAELs/NOAELs of 7/0.7 mg/kg/day for 4-MBC and 0.24/0.07 mg/kg/day for 3-BC. Molecular endpoints were affected by the lowest doses. In order to obtain information on human exposure, we conducted a monitoring study on human milk with three series of mother–child pairs (2004, 2005, 2006), with focus on cosmetic UV filters in relation to other endocrine disruptors. Methods for UV filter analysis followed the principles of European standardized methods for pesticide residue analysis (EN 15289). In cohorts 2004 and 2005, 78.8% of women reported use of product(s) containing cosmetic UV filters in a questionnaire, and 76.5% of milk samples contained these filters. Use of UV filters and concentration in human milk were significantly correlated. The results agree with the idea of transdermal passage of UV filters. They also indicate that it may be possible to reduce human exposure during critical periods such as pregnancy and lactation by transiently abstaining from use.

**Keywords:** 3-Benzylidene camphor (3-BC) · Developmental toxicity · Human milk · 4-Methylbenzylidene camphor (4-MBC) · UV filters

## 1. Endocrine Activity of UV Filters

UV filters are either physical filters like titanium dioxide and zinc oxide, which mainly scatter and reflect UV rays, or organic molecules absorbing light in the UV range (UVA 400–320 nm, UVB 320–280 nm). These organic compounds often possess single or multiple aromatic structures capable of absorbing energetic solar photons and returning to the ground state by thermally emitting the absorbed energy.<sup>[1]</sup> Only substances listed in cosmetic directives like EU Cosmetics Directive, Swiss Ordinance for Cosmetics, are allowed for use in sunscreens and as additives in cosmetics. Currently 27 UV filters are permitted for cosmetic use in Europe. In spite of considerable structural similarities with authorized cosmetic UV filters, technical UV filters in plastics and other products need not be declared.

Since the introduction of cosmetic UV filters, the main concern regarding their

use was the efficiency to protect human skin from adverse effects of UV light while avoiding dermatological side effects. Observations made in the 1990s on penetration of human skin by UV filters,<sup>[2,3]</sup> and on their presence in fish<sup>[4]</sup> indicated the possibility of systemic effects of these chemicals and prompted us to investigate their endocrine activity. When tested on MCF-7 cells *in vitro*, a number of UV filters used in sunscreens exhibited estrogenic activity; some also stimulated growth of the immature rat uterus in a short-term *in vivo* test for estrogenic activity.<sup>[5]</sup> Estrogenic activity of UV filters has subsequently been confirmed in several *in vitro* and *in vivo* tests on mammals and fish.<sup>[6–14]</sup> Certain UV filters also display anti-androgenic activity *in vitro*,<sup>[15,16]</sup> and can affect the thyroid axis.<sup>[17–19]</sup>

Two UV filters with comparatively high estrogenic activity are 4-methylbenzylidene camphor (4-MBC) and 3-benzylidene camphor (3-BC).<sup>[5,12]</sup> Both compounds exhibit ER beta preference, but they are also active

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in ER alpha, typically in *in vivo* tests such as the uterotrophic assay, 4-MBC possibly because of a hydroxylated metabolite.<sup>[20]</sup> Whereas a 90-day dermal exposure study in adult rats reported to the Scientific Committee on Consumer Products<sup>[21]</sup> failed to disclose estrogenic effects of 4-MBC in this chronic model, significant estrogenic and antiestrogenic effects were observed in 90-day studies on adult ovariectomized rats that addressed this question more specifically. Typical estrogen targets affected by 4-MBC include luteinizing hormone, leptin, fat depots, bone, and genes such as insulin-like growth factor-1.<sup>[22,23]</sup> Interactions with estrogenic mechanisms were also observed after three months exposure to benzophenone-2.<sup>[24,25]</sup> Effect patterns of UV filters do not fully mimic those of natural estrogens, which may be explained, *i.a.*, by partial agonist activity, different affinities for estrogen receptor (ER) alpha and ER beta, or interactions with different hormone axes.<sup>[5,12,17]</sup>

## 2. Developmental Toxicity of 4-Methylbenzylidene Camphor (4-MBC) and 3-Benzylidene Camphor (3-BC)

Development of the sexual phenotype depends on the intervention of sex hormones. In mammals, testosterone (T) directs development of the male, but this action is not exclusively mediated by androgen receptors (AR). In brain, T is converted to estradiol (E2)<sup>[26,27]</sup> to control male differentiation. Observations in aromatase knockout mice indicate that development of the female brain also depends on E2.<sup>[28]</sup> Certain peripheral male tissues such as prostate convert T to dihydrotestosterone acting on AR as well as to E2 acting on ER.<sup>[29,30]</sup> With the exception of tissues with local conversion of T to E2, endogenous estrogen levels are very low in fetuses of both sexes in rodents<sup>[31]</sup> and also comparatively low in humans,<sup>[32]</sup> thus facilitating competition of weak estrogenic chemicals for ER.

### 2.1. Treatment Design

Potential effects of exposure to 4-MBC and 3-BC during pre- and postnatal development were investigated in a mammalian model, the rat.<sup>[17,19,33–35]</sup> Male and female rats of the parent generation were exposed to 4-MBC (47, 24, 7.0, 0.7 mg/kg/day) or 3-BC (7.0, 2.4, 0.7, 0.24, 0.07 mg/kg/day) administered in chow at least ten weeks before mating, females were further treated during pregnancy and lactation, and their F1 offspring until adulthood (age of three months). 47 mg/kg/day 4-MBC corresponds to 40% of uterotrophic LOAEL, 3-BC was dosed according to uterotrophic potency relative to 4-MBC.<sup>[12]</sup> Schauer *et*

*al.*<sup>[36]</sup> suggested that toxicity of 4-MBC should be studied with dermal application. Yet, certain processes of sexual differentiation like sexual brain differentiation have long been known to be highly sensitive to pre- and postnatal stress and handling,<sup>[37]</sup> so that results obtained with topical application of chemicals to the skin of F0 and F1 rats, each for three months, would be expected to yield questionable results.

### 2.2. 4-MBC and 3-BC in Rat Milk

For comparison of experimental data with internal human exposure, the chemicals were determined in rat milk taken from the stomach on postnatal day 6. Concentrations (ng/g lipid) were as follows (mean  $\pm$  SD, number of samples/litters): 4-MBC, 7 mg/kg/day: 208.6  $\pm$  108.7 (5/5), 0.7 mg/kg/day: 86.3  $\pm$  40.5 (4/3), 3-BC, 0.24 mg/kg/day: 132.1  $\pm$  75.2 (3/3), 0.07 mg/kg/day: not detectable (4/4). 4-MBC and 3-BC were undetectable in milk of controls.

### 2.3. Postnatal Development

In the higher dose range, both UV filters reduced survival rate.<sup>[17,38]</sup> An impairment of the developing immune system was indicated by a decrease in thymus weight at postnatal day (PN) 14. A prominent effect of both UV filters during early postnatal development is the significant delay of puberty in males (preputial separation). Puberty onset in females remained unaffected (Tables 1, 2<sup>[17,34]</sup>). 4-MBC and 3-BC thus mimic the typical effect of E2 only in males, but differ from E2 in females, where E2 advances puberty.<sup>[39]</sup> Body weight at puberty was slightly reduced in females but normal in males, indicating that the delay of male puberty did not result from nutritional effects. Adult body weights were at control level after 4-MB<sup>[19,33,34]</sup> and 3-BC except for a slight reduction after the highest dose of 3-BC, possibly as a result of estrogenic activity.<sup>[39]</sup>

### 2.4. Low-dose Effects in Neonatal Uterus and Prostate

Estrogen target gene mRNA levels in early postnatal uterus (PN 6), determined by real-time RT PCR,<sup>[33]</sup> were affected at doses as low as 0.07 mg/kg/day 3-BC and 0.7 mg/kg/day 4-MBC (Table 2). The same doses also affected gene expression in sexually dimorphic brain regions at PN 6 (M. Fuetsch, C. Gaille, unpublished data). The changes in mRNAs encoding for vascular epithelial growth factor (VEGF), inducible nitric oxide synthase (iNOS) and, in part, endothelial cell nitric oxide synthase (ecNOS) suggest that angiogenesis and blood flow in uterus may be influenced. Effects on male accessory sex glands were investigated by morphometric analysis in collaboration with L. Hofkamp and B. Timms. Maternal exposure to 4-MBC re-

sulted in significant increases in the size of prostate, seminal vesicles and coagulating gland at PN 1 (day of birth). In line with other findings on estrogenic chemicals, marked differences in growth responses of specific regions of the prostate were observed (Table 1, Hofkamp *et al.*<sup>[40]</sup> and unpublished data).

### 2.5. Reproductive Organs of Adult Offspring: Regulation of Target Gene Expression

Exposure to 4-MBC and 3-BC affected reproductive organ weights (Tables 1, 2<sup>[17,33,34]</sup>). Testes of 4-MBC-exposed offspring showed decreased weight at PN14<sup>[38]</sup> and increased relative weight at the highest dose in adulthood. The adult finding is reminiscent of neonatal administration of weak estrogens.<sup>[41,42]</sup> In contrast, the decrease in prostate weight of 4-MBC-exposed offspring resembles the effect of perinatal administration of the potent ER agonist diethylstilbestrol.<sup>[41,43]</sup> This suggests a differential sensitivity of male target organs.

The same estrogen target genes were studied in reproductive organs and brain of male and female offspring in order to compare effects on gene regulation. In ventral and dorsolateral prostate and uterus, gene expression was affected at mRNA and protein levels in a tissue-specific manner.<sup>[33,34]</sup> The 4-MBC-induced decrease in prostate weight was accompanied by a decrease in AR, ER alpha, and insulin-like growth factor-I (IGF-I) (Table 1). In 4-MBC-exposed uterus, affected target genes include ER alpha and progesterone receptor (PR) (Table 2). Effect patterns differed between the two camphor derivatives also at the molecular level, in spite of close structural relationship and similar actions in acute assays for estrogenicity.

Malfunctions may also be caused by changes in the sensitivity of tissues to natural estrogens. In order to assess such changes, 4-MBC-exposed offspring were gonadectomized in adulthood, injected two weeks later with a single dose of E2 (10 or 50  $\mu$ g/kg s.c.), and investigated 6 h after the injection. 4-MBC exposure reduced the acute up-regulation of PR and IGF-I and down-regulation of ER alpha and AR mRNA by E2 in uterus, and the down-regulation of AR and IGF-I mRNA by E2 in ventral prostate (Table 1<sup>[33,34]</sup>). The reduced up-regulation of estrogen target genes in uterus was accompanied by decreased steroid receptor coactivator-1 (SRC-1) protein levels, while reduced down-regulation of genes in prostate was paralleled by reduced nuclear receptor corepressor (N-CoR) protein.<sup>[33,34]</sup> This identifies nuclear receptor coregulators as targets of endocrine receptors, and suggests that they are involved in changes in estrogen sensitivity.

Table 1. Effect of 4-MBC and 3-BC on selected endpoints in male rat offspring

	4-Methylbenzylidene camphor [mg/kg/day p.o.]				3-Benzylidene camphor [mg/kg/day p.o.]				
	0.7	7	24	47	0.07	0.24	0.7	2.4	7
<b>Puberty</b> (preputial separation)		Delayed	Delayed	Delayed		∅	∅	Delayed	Delayed
<b>Adult body weight</b>	∅	∅	∅	∅	∅	∅	∅	∅	↓
<b>Testis</b>									
Postnatal Day 14, Testis relative weight		↓	↓	↓					
Adult F1, Testis relative weight	∅	∅	∅	↑		∅	∅	∅	∅
<b>Prostate</b>									
Postnatal Day 1, Duct number (dorsal) and duct volume (ventral)	∅	↑	↓	↓	∅	∅	∅	∅	∅
Adult F1, Ventral lobe relative weight	∅	↓	↓	↓	∅	↓	∅	∅	∅
<b>Gene expression, adult F1 prostate, mRNA/protein</b>									
Androgen receptor (AR) dorsolateral prostate (DP)	∅/∅	↓/∅	↓/↓	↓/(↓)		↑/↓	∅/↓	∅/-	↑/-
Androgen receptor (AR) ventral prostate (VP)	∅/∅	∅/(↓)	↓/↓	∅/-		↑/↑	↑/∅	↑/-	∅/-
AR mRNA down-regulation by estradiol in VP		↓	↓						
N-CoR protein, DP	↓	↓	(↓)			↓	↓		
N-CoR protein, VP	∅	(↓)	↓			∅	∅		
<b>Central nervous system, adult F1, mRNA</b>									
Gene expression in Ventromedial Hypothalamic Nucleus									
Estrogen Receptor-alpha		∅	↓	↓		↑	↑	∅	∅
Progesterone Receptor		∅	∅	∅		∅	∅	↑	↑

↑, ↓: significant increase or decrease *versus* control for p < 0.05 or better. ∅: no statistically significant change. blank or -: not analyzed.  
 PN 1 = day of birth. Adult F1 offspring: 12 weeks of age, studied under baseline conditions.  
 Data from Schlumpf *et al.* [17, 38], Durrer *et al.* [34], Maerkel *et al.* [19], Hofkamp *et al.* [40], O. Faass, M. Fuetsch, C. Ehnes, C. Gaille, unpublished data.

Table 2. Effect of 4-MBC and 3-BC on selected endpoints in female rat offspring

	4-Methylbenzylidene camphor [mg/kg/day p.o.]				3-Benzylidene camphor [mg/kg/day p.o.]				
	0.7	7	24	47	0.07	0.24	0.7	2.4	7
<b>Puberty</b> (vaginal opening)		∅	∅	∅		∅	∅	∅	∅
<b>Adult body weight</b>	∅	∅	∅	∅	∅	∅	∅	∅	↓
<b>Ovary</b>									
Adult F1, Ovary relative weight	∅	∅	↑	↑					
<b>Uterus</b>									
Postnatal Day 6, Uterus relative weight	∅	∅			∅	∅			
Adult F1, Uterus relative weight	∅	∅	↑	∅		∅	∅	∅	↓
<b>Postnatal Day 6 Uterus, Gene expression, mRNA</b>									
Estrogen receptor-alpha	↓	∅			↓	↓			
ecNOS	∅	∅			↓	(↓)			
iNOS	↓	↓			↓	↓			
VEGF	↓	∅			↓	↓			
<b>Adult F1 Uterus, Gene expression, mRNA/protein</b>									
Progesterone Receptor (PR-A protein)	∅/↓	∅/∅	↓/∅	↓/∅		∅/↑	∅/∅	∅/∅	↑/∅
PR mRNA up-regulation by estradiol	∅	↓	↓						
SRC-1 protein	↓	∅	(↓)	↓		∅	(↓)	↓	∅
<b>Central nervous system, adult F1, mRNA</b>									
Gene expression in Ventromedial Hypothalamic Nucleus									
Estrogen Receptor-alpha		↓	↓	↓		∅	∅	↑	↑
Progesterone Receptor		↓	↓	↓		↑	∅	↓	↓
PR mRNA up-regulation by estradiol		↑	∅						
<b>Female sexual behavior (proceptive and lordosis behavior)</b>		↓	↓					↓	↓
<b>Estrous cycle</b>		∅	∅	∅		irregular	irregular	irregular	irregular

↑, ↓: significant increase or decrease *versus* control for p < 0.05 or better. ∅: no statistically significant change. blank: not analyzed.  
 PN 1 = day of birth. Adult F1 offspring: 12 weeks of age, studied under baseline conditions, females in diestrus.  
 Data from Schlumpf *et al.* [17, 38], Durrer *et al.* [33], Maerkel *et al.* [19], O. Faass, M. Fuetsch, C. Ehnes, C. Gaille, unpublished data.

## 2.6. Sexually Dimorphic Gene Expression in Brain and Female Sexual Behavior

In consideration of the data on brain differentiation and fetal estrogen levels as outlined above, we hypothesized that the female brain should be sensitive to estrogenic chemicals. Female sexual behavior was recorded in adult female offspring exposed to one of two doses of 4-MBC (7, 24 mg/kg/day) or 3-BC (2.4, 7 mg/kg/day). Exposed females were mated with normal experienced males in the evening of proestrus, at the onset of the dark phase (16.00), when behavioral receptivity of gonadally intact rats is high.<sup>[44]</sup> Behavior was recorded on coded videotapes in a room illuminated by an infrared light source. Vaginal smears were recorded for at least 10–14 days before behavioral testing. All four treatments strongly suppressed female sexual behavior (Table 2, Faass *et al.*, unpublished data). The treatments affected proceptive behavior (jumping and ear wiggling, displayed to attract the male), as well as receptive (lordosis) behavior (decreased lordosis quotient  $LQ = \text{number of lordosis responses/number of mounts} \times 100$ ). At the same time, the male attempting to mount was rejected more frequently by the female (rejection behavior). Detailed analyses of estrous cycles were performed on additional groups of animals for 21 and 16 days in 4-MBC- and 3-BC-exposed offspring, respectively. In 4-MBC-exposed offspring, female sexual behavior was disturbed in the presence of normal estrous cycles, whereas 3-BC exposure caused irregular cycles. The two functions thus are differentially affected.

Gene expression was analyzed by real-time RT PCR in adult male and female offspring in two brain regions involved in the control of gonadal function and sexual behavior, medial preoptic region (MPO) and ventromedial hypothalamic nucleus (VMH) (Tables 1, 2, 4-MBC:<sup>[19,35]</sup> 3-BC: Faass *et al.*, unpublished data). Both compounds caused sex- and region-specific changes in ER, in nuclear receptor coactivator SRC-1, and in target gene mRNA levels. A drop of PR mRNA in female VMH down to male levels emerged as a common feature observed after all doses of 4-MBC and after the higher two doses of 3-BC (tested for behavioral effects). Reduced PR mRNA in female VMH was correlated with impaired female sexual behavior. A similar relationship had been observed with a polybrominated flame retardant (PBDE 99) and with a PCB mixture.<sup>[45]</sup> Lordosis behavior is directly correlated with the expression of PR mRNA in VMH of female rats.<sup>[46,47]</sup> Loss of sexual dimorphism of PR in female VMH thus appears to represent a signal of altered regulation of PR that is linked with behavioral impairment across different endocrine disruptors.

## 3. UV Filters in Environment and Food Chain

There is good evidence that pharmaceuticals and ingredients of personal care products (PPCPs) can spread to the biosphere and reach the food chain. UV filters may be directly introduced into surface waters during swimming or may enter wastewater from households or industry at several levels of industrial production or commercial use.<sup>[1]</sup> Cosmetic compounds, synthetic perfumes and UV filters, were detected in high amounts in Swiss sewage sludge.<sup>[48]</sup> UV filters and synthetic musks are present in surface waters and in biota at various trophic levels, in particular in fish.<sup>[4,49–52]</sup> UV filter levels in fish from rivers receiving inputs from wastewater treatment plants (WWTPs) had considerably higher chemical loads than fish from Swiss lakes with inputs from WWTPs,<sup>[50]</sup> suggesting increased availability of these contaminants for fish in rivers. These studies identified WWTPs as a major source for UV filters in the aquatic environment and demonstrate the presence of UV filters in the food chain.

## 4. Human Exposure: Monitoring of Human Milk

Assessment of chemical risks requires information on quality and quantity of chemicals present in human body during critical and sensitive life stages such as pre- and postnatal development. Acute and short-term (4 d) experiments with percutaneous application of 4-MBC to human volunteers indicated transdermal passage of the compound,<sup>[36,53]</sup> but such studies do not yield information on internal exposure of the human population under realistic patterns of cosmetic use. This information can be provided by analysis of human milk, which informs on internal exposure of mother and fetus and on contamination of the food provided to the nursing infant. Most of the existing data relate to organochlorine compounds. Unfortunately, their trend to decrease in human milk has been considered as a success in the campaign against chemical exposure of babies, without asking for possible exposures to other chemicals from the food web, like cosmetics, pharmaceuticals, industrial or household products. Thus, phthalate exposure has recently been linked with alterations in male genital development and hormone profiles.<sup>[54,55]</sup>

## 5. The Swiss Cohort

Since there was no information on internal exposure of human populations to UV filters, and very limited information on ad-

ditional cosmetic ingredients, we started a monitoring study of human milk at the University Hospital Basel with the approval of the Basel University Ethics Committee. The study focused primarily on chemical analysis of UV filters in relation to several other groups of endocrine-disrupting chemicals (EDCs and EDC candidates) and consisted of three different cohorts over three years (2004, 2005, 2006). So far, the first two cohorts have been evaluated. For the first time the questionnaire given to the mothers contained very detailed questions on the use of cosmetics in pregnancy and lactation. The aim was to detect a possible correlation between exposure to certain UV filters and their presence in human milk.

### 5.1. Questionnaires

All mothers had to fill out a questionnaire and to give written consent for participation in the study. The questionnaires asked for mother and child data on birth date, sex, height, weight, sisters and brothers, education, professional career, living area (urban, suburban, rural), nutritional and smoking habits of mothers. The questionnaire then asked for detailed qualitative and semiquantitative (daily, weekly, monthly or less) use of different types and brands of cosmetic products during pregnancy and lactation, including sunscreens, lipsticks, perfumes, deodorants, skin care creams, body lotions, shower lotions, bubble baths, hair dyes, make-ups *etc.*

### 5.2. Sampling of Human Milk

Sampling was supervised by Claudia Vökt with the assistance of the study nurse Monika Birchler. Care was taken to avoid contamination. The mothers were instructed to clean breast and nipples thoroughly with warm tap water before milk sampling. The milk was obtained using a freshly hot water-rinsed milk pump (Type Harmony, Medela AG, Baar). The milk servings were collected in a clean sterilized bottle (Schott Duran ISO 4796) stored in the freezer at  $-20^{\circ}\text{C}$ . Milk sampling mainly included the transitory phase of lactation (day 6 to 14 after birth), occasionally also the first days of the mature phase of lactation (from 14 days after birth on), rarely the colostrum phase (first 6 days after birth) (Wünschmann *et al.*<sup>[56]</sup>). The numbers of milk servings per bottle (around 100 ml) representing the individual milk sample of each mother taken for chemical analysis, varied between 4 and 10.

### 5.3. Chemicals Analyzed

Our intention was to simultaneously analyze different groups of EDCs in order to obtain information on their relative importance. Together with Karin Kypke from the Community Reference Laboratory for Pesticides in Food of Animal Origin at the

State Institute for Chemical and Veterinary Analysis of Food in Freiburg/Germany, we analyzed eight of a total of 27 authorized cosmetic UV filters (Tables 3, 4) in the same human milk sample, as we wanted to know whether these cosmetic UV filters concentrate in human milk, and whether there is a correlation between use of cosmetics containing these chemicals and their presence in human milk. The same milk samples were also analyzed for synthetic fragrances such as nitro musks (musk xylene, musk ketone), polycyclic musks including HHCB (Galaxolide) and AHTN (Tonalide), macrocyclic musks, several polybrominated diphenylethers (BDE28, 47, 99, 100, 153, 154), organochlorine pesticides (including DDT/DDE, methoxychlor, hexachlorocyclohexane (HCH), hexachlorobenzene, toxaphene), seven indicator PCB congeners, and cyclodiene insecticides (aldrin, dieldrin, chlordane, endrine, endosulfan, heptachlor bromocyclene) (Schlumpf *et al.*, in preparation).

#### 5.4. Extraction of UV Filters

The amount of human milk used as a sample depends on the lipid content of the milk. Routinely a sample amount of 0.25–0.5 g lipid per sample was analyzed. Human milk samples were centrifuged and the UV filters of interest (Table 3) were extracted from the cream together with lipid, using sodium sulfate and the solvent n-hexane/acetone (1:1) at first, followed by dichloromethane/acetone (1:1). Following evaporation of the solvent in a rotary evaporator, the extract containing the UV filters was re-dissolved in cyclohexane/ethyl acetate (1:1), centrifuged and three internal standards for the eight UV filters

were added. To remove lipid, gel permeation chromatography was performed on Bio-Beads S-X3 with cyclohexane/ethyl acetate as eluting solvent. The eluate was concentrated to a defined volume. Analogous procedures were used for additional groups of lipophilic xenobiotic substances analyzed in the same milk sample, like persistent organochlorine compounds, synthetic musks and PBDEs. These data will be presented in the final report on all three cohorts (Schlumpf *et al.*, in preparation).

In rats, the whole stomach of the pup was homogenized and extracted using acetone and n-heptane in a Dispomix homogenization system (Medic Tools). After adding the internal standards, the sample was shaken 15 min at 0 °C. and centrifuged, the supernatant collected and dried. The extraction step was repeated and the UV filters were separated from lipids by RP-HPLC using a octadecylsilyl column (Zenker *et al.*, in preparation). The fraction containing 4-MBC and 3-BC was dried in a vacuum centrifuge, re-dissolved in ethanol and determined by GC-MS (see below).

#### 5.5. Determination of UV Filters

The method for analysis of UV filters in human milk samples followed the principles of the European standardized methods for pesticide residue analysis.<sup>[57]</sup> Determination of all UV filters except Bp-2 was done by GC-LRMS (GC: HP 6890; MS: HP 5973; 30 m HP5-MS, 0.25 mm i.d., 0.25 µm film thickness + 2.5 m pre-column) with MSD-EI detection mode, using selected ion monitoring (SIM mode) and selecting one target and three qualifier ions as characteristic mass ions. To compensate for matrix effects matrix-matched calibra-

tion was used. For determination of Bp-2 LC-MSD (LC: HP 1100; MS: Quattro LC, 50 × 2 mm Luna C18 (2), 5 µm Phenomenex) with the detection mode ESI pos was applied, using multiple reaction monitoring (MRM), eluent: A being 1 mM ammonium acetate, pH 4.75 and eluent B being methanol, using matrix-matched calibration. The concentrations of the substances are reported as ng per g of milk lipid (ng/g lipid). The limit of quantification (LOQ) and the limit of determination (LOD) for the UV filters: HMS, 3-BC, BP-3, 4-MBC and OC were 4.0 (ng/g lipid) (for LOQ) and 2.0 (ng/g lipid) for LOD. For OD-PABA, EHMC and BP-2, LOQ was 2.0 ng/g lipid and LOD 1.0 ng/g lipid. The mean level for each residue was calculated with the assumption of zero level for undetected value and half LOQ for levels determined between LOD and LOQ. The level was stated as 'nd', *i.e.* undetected, if it was below LOD.

#### 5.6. Biostatistics

Possible relationships between use of UV filters and chemical-analytical data in human milk were analyzed by Valentin Rousson, Biostatistics Unit, University of Zurich, using Pearson Chi-Square and Fisher's exact test.

#### 5.7. Use of UV Filters in Cosmetics and Presence in Human Milk

An analysis of the first two cohorts from the Basel cohort study, pilot study (2004) and Study 1 (2005), revealed that during the periods of pregnancy and lactation, 78.8% of the women used some cosmetic product containing UV filters. In 76.5% of human milk samples, UV filters were detected (Table 4). Ethylhexyl-methoxycinnamate (EHMC, previously known as octyl-methoxycinnamate (OMC)) and octocrylene (OC) were the UV filters most frequently used according to the questionnaire and most frequently detected in milk samples. For these two filters, a significant correlation between use and presence in human milk could be demonstrated for the individual chemical ( $p = 0.031$  for EHMC,  $p = 0.046$  for OC, Fisher's exact test). The correlation was also significant across all UV filters for use and presence in the corresponding milk sample ( $p = 0.009$ ). Interestingly, only 45.5% of women reported use of sunscreens with UV filters, whereas 60.6% of the women used other cosmetics containing UV filters.

These data demonstrate concentration of UV filters in a relevant proportion of human milk samples. Except for lipsticks where oral uptake is probably important, these results agree with the idea of transdermal passage of UV filters from cosmetics, as proposed from animal and human studies.<sup>[15,36,53,58]</sup> However, it should be kept in mind that there are also other sources for

Table 3. UV filters analyzed in human milk

Abbreviation	Chemical	INCI <sup>a)</sup> Nomenclature	Purity of reference chemical
Bp-2	2,2',4,4'-Tetrahydroxi-benzophenone	Benzophenone-2	97%
Bp-3	2-Hydroxi-4-methoxi-benzophenone	Benzophenone-3	98%
3-BC	3-Benzylidene-bornane-2-on	3-Benzylidene Camphor	> 97%
4-MBC	3-(4'-Methyl)benzylidene bornane-2-on	4-Methylbenzylidene Camphor	> 99.7%
EHMC (OMC)	2-Ethylhexyl-4-methoxycinnamate	Ethyl-hexylcinnamate (Octyl-methoxycinnamate)	98%
HMS	3,3,5-Trimethyl-cyclohexyl-salicylate, Homosalate	Homosalate	> 98%
OC	2-Cyano-3,3'-diphenyl-acrylic acid 2'-ethyl-hexylester	Octocrylene	98%
OD-PABA	4-Dimethylamino-benzoic acid-2 ethyl-hexyl-ester	Octyl-dimethyl PABA	> 98.5 %

<sup>a)</sup>INCI: International Nomenclature of Cosmetic Ingredients

Table 4. UV filters in human milk<sup>a</sup>

	Percentage of women using product with compound n = 34 % of Total	Percentage of milk samples with compound n = 34 % of Total	Levels in Human Milk		
			Mean ± SD, number of positive samples ng/g lipid	Median ng/g lipid	Range in positive samples ng/g lipid
<b>UV filters</b>					
EHMC / OMC <sup>b</sup>	58.8	64.7	28.9 ± 22.1 (22)	25.0	2.1 - 78.1
Octocrylene	38.2	47.1	18.3 ± 17.97 (16)	12.5	4.7 - 77.5
Bp-3	14.7	18.2	49.2 ± 54.9 (6)	19.8	7.3 - 121.4
4-MBC	17.7	11.8	15.6 ± 5.9 (4)	18.4	6.7 - 19.0
OD-PABA	2.94	2.94	50.0 ± 0 (1)	50.0	50.0
HMS	14.7	0	n.d.	n.d.	n.d.
Bp-2	11.8	0	n.d.	n.d.	n.d.
3-BC <sup>c</sup>	0.0	0	n.d.	n.d.	n.d.
UV Filters in sunscreens	45.5				
UV filters in different cosmetics	60.6				
% of women using any product with UV Filters	78.8	76.5			
% of milk samples containing any of the UV filters					

<sup>a</sup>Combined data from Pilot Study (2004, n = 13) and Study 1 (2005, n = 21). Study 2 (2006, n = 20) not yet incorporated.

<sup>b</sup>Abbreviations: EHMC = ethylhexylmethoxy cinnamate = OMC = octylmethoxy cinnamate, 4-MBC = 4-methylbenzylidene camphor, 3-BC = 3-benzylidene camphor, Bp-3 = benzophenone-3, Bp-2 = benzophenone-2, HMS = homosalate, OD-PABA = octyldimethylamino benzoic acid.

<sup>c</sup>Authorised for use in cosmetics in 2006.

these compounds, since they are present in the ecosphere and food chain (see above). The Basel cohort study shows multiple chemical exposures of neonates to groups of identified and candidate EDCs. According to these results, it is evident that exposure (use) of cosmetics containing UV filters will produce UV filter-positive human milk samples. Abstinence from use of organic UV filter containing sunscreens and cosmetics could therefore be considered as an important step to diminish the total load of chemicals in human milk, in order to reduce exposure during particularly sensitive life stages.

### 6. Comparison of Experimental Rat Data with Human Exposure

Our data indicate that pre- and postnatal exposure to 4-MBC and 3-BC can interfere with sexual development at brain and reproductive organ levels. Classical toxicological endpoints such as puberty and reproductive organ weights exhibited lowest observed adverse effect lev-

els (LOAEL) and no observed adverse effect levels (NOAEL) of 7 and 0.7 mg/kg/day for 4-MBC and of 0.24 and 0.07 mg/kg/day for 3-BC, respectively. Molecular endpoints were affected by the lowest doses studied. At the LOAEL of 7 mg/kg/day, 4-MBC concentration in rat milk (208.6 ng/g lipid) was eleven times the highest value so far found in human milk (19 ng/g lipid, Table 4). The ratio at the classical NOAEL and molecular LOAEL of 0.7 mg/kg/day 4-MBC is 4.5. This comparatively small ratio indicates that the potential risk posed by UV filters warrants further considerations.

#### Acknowledgements

The investigations were supported by Swiss National Research Program 50, EU 5th Framework Program (EURISKED), Swiss Federal Office for the Environment, Hartmann-Müller Stiftung, and the Olga Mayenfisch Stiftung.

Received: April 2, 2008

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