Investigation of human exposure, metabolism and biological effects of the antibacterial triclocarban

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1 INTRODUCTION

Triclocarban (TCC, 3,4,4’-Trichlorocarbanilide) has been widely used as an antibacterial agent in personal care products for more than 45 years (Chen et al, 2008). Many of the antimicrobial bar soaps sold in the US contain up to 1.5% of TCC, amounting to about a million pounds of this chemical being used every year (Halden & Paull, 2004; Halden & Paull, 2005). In Figure 1 a few representative examples of antibacterial soaps from the US market are shown alongside with the chemical structure of TCC. Albeit TCC is a legal additive in Germany and the European Union, personal care products (PCP) containing TCC are rarely found on the German market.

![Fig. 1: Antibacterial soaps containing triclocarban (TCC) from the US market and the chemical structure of TCC.](image)

The antimicrobial mechanism underlying the bacteriostatic and bactericidal effects of TCC is assumed to be an unspecific adsorption to cell membranes and interruption of their function. As a consequence, the growth of gram-positive as well as gram-negative bacteria is inhibited (European Union, 2005). However, the beneficial antibacterial effect for human health is controversial. For example, antibacterial and plain soaps show a similar effectivity in preventing infectious diseases (Luby et al, 2005). This suggests that the physical removal of pathogens from hands and skin...
with soap and water, rather than the antibacterial activity of soap, is the key factor in the prevention of diarrhoea, impetigo, and respiratory infections (Luby et al, 2005).

Due to its frequent use, levels of TCC of up to 5 ppb (16 nM) have been detected in surface water in the United States (Halden RU, 2004; Halden RU, 2005). The Targeted National Sewage Sludge Survey published in 2009 by the US Environmental Protection Agency (EPA) reported the presence of TCC in all 84 sludge samples analyzed. TCC was found at a concentration of up to 0.44 g/kg, indicating a strong accumulation of TCC in sludge (EPA, 2009). Moreover, this study showed that TCC together with the other frequently used antibacterial agent triclosan was present in the samples at the highest concentration of all synthetic compounds screened for.

Several studies reported the bioaccumulation of TCC in aquatic organisms. Studies on the TCC concentration in water, algae (Cladophora spp.) and snail (Helisoma trivolvis) collected downstream of a wastewater plant calculated a bioaccumulation factor (BAF) of 1600-2700 (log BAF 3.2-3.4) in both organisms (Coogan et al, 2007; Coogan & La Point, 2008). The freshwater worm Lumbriculus variegatus absorbs TCC directly from sludge (Higgins et al, 2009). For most aquatic organisms that might enter the human food chain, no data regarding the bioaccumulation of TCC are available. However, several studies indicate that the BAFs are similar among different aquatic species (Kenaga, 1980a) and generally show a good correlation with the physicochemical properties of the compounds, e.g. the $n$-octanol-water-coefficient ($K_{ow}$) (Kenaga, 1980a; Kenaga, 1980b). Thus, bioaccumulation in fish or even trophic accumulation along the food chain might occur. Besides the risk derived from the consumption of contaminated food, dermal exposure due to the use of TCC containing PCP is the primary route of human exposure (see below).

In the 1980s, a few studies were carried out on human exposure to TCC. These indicated that bathing with TCC-containing soaps typically results in a deposition of TCC on human skin of $\approx$0.3 µg/cm$^2$ (North-Root et al, 1985; North-Root et al, 1984).
Based on studies with $^{14}$C-labelled TCC it was demonstrated that a low but significant portion of TCC in soaps is percutaneously absorbed by humans during and after bathing and showering (Scharpf et al, 1975). *In vivo* studies in rodents, monkeys and man revealed that TCC is rapidly metabolized via different pathways. The main metabolite found in human urine was suggested to result from its direct glucuronidation (Birch et al, 1978; Hiles & Birch, 1978a; Hiles & Birch, 1978b), accounting for 25% of TCC elimination from the body. Most of the absorbed TCC is metabolized by enzymes of the cytochrome P450 (CYP) superfamily to different hydroxylated TCC species (phase I metabolites), which are subsequently conjugated with glucuronic acid or sulfated and predicted to be excreted via the feces (Birch et al, 1978; Hiles, 1977; Hiles & Birch, 1978a; Hiles & Birch, 1978b; Jeffcoat et al, 1977; Warren et al, 1978). The main phase I metabolites are 2'-OH TCC and 6-OH TCC, both bearing the hydroxyl group in the *ortho* position relative to the urea group. Additionally, 3'-OH TCC and dihydroxylated metabolites, including 2',6-diOH TCC and 3',6-diOH TCC, are formed (Birch et al, 1978; Warren et al, 1978). Acute toxicity of TCC in rodents was found to be low. Regardless of the administration route, no signs of toxicity were reported up to a concentration of 2 g/kg body weight in rodents (BW) (EuropeanUnion, 2005). Based on these data and other earlier toxicological studies, the Scientific Committee on Consumer Products of the European Commission regarded the use of TCC in personal care products as safe (EuropeanUnion, 2005).

Recent findings indicate a significant, unintended biological activity. By enhancing the action of steroids, TCC has the potential to act as an endocrine disruptor at high concentrations by enhancing the action of testosterone and other steroids (Ahn et al, 2008; Chen et al, 2008). Based on its high persistence in the environment and the direct exposure of humans through PCP, TCC might be able to influence human health.
2 Scope

Recent studies demonstrate that triclocarban (TCC) accumulates in the aquatic environment and is the most abundant synthetic compound present in sewage sludge. Moreover, the antibacterial agent possesses unrecognized off-target properties, which might affect human health and mammalian biology, e.g. by acting as an endocrine disruptor. The aim of the present work was to perform a safety evaluation of TCC. The first step to assess potential health risks is the determination of human exposure levels. Therefore, we investigated the exposure resulting from bathing with TCC containing soaps (section 3.1.1). Since two independent $^{14}$C exposure studies in man showed that a relevant portion (25%) of the TCC absorbed is excreted via the urine (Hiles & Birch, 1978a; Scharpf et al, 1975), non-invasive urine sampling together with the quantification of the compound in blood (section 3.1.2) was used to determine human exposure. Several studies indicate that accumulation according to $n$-octanol-water-coefficient ($K_{OW}$) in aquatic species such as algae does in fact occur. However, no data on higher organisms were available. Therefore, we analyzed in section 3.2 whether TCC accumulates in fish and has the potential to a trophic accumulation, leading to an enhanced risk of contaminating food. The assessment of the human as well as the environmental exposure requires analytical methods for the quantification of TCC and its metabolites in a large number of samples. Therefore, the aim of section 3.2 was to develop two methods for the quantification of TCC in complex biological samples such as blood and urine. Here two orthogonal techniques, liquid chromatography (LC) electrospray ionization (ESI) mass spectrometry and an immunoassay, which does not require expensive lab equipment, were used. Metabolism is the crucial step in the elimination of lipophilic compounds such as TCC. Therefore, in section 3.3 TCC’s phase I and phase II metabolism was thoroughly (re)-evaluated by means of modern analytical chemistry methods. A focus was set on the investigation of a potential toxification by metabolic reactions and characterization of reactive metabolites. In section 3.4, the non-intended biological activity of TCC on mammalian enzymes was investigated. The potential systemic effects of exposure on affected enzymatic pathways in mammalian biology are assessed in vivo by means of targeted oxylipin metabolomics. Finally, the implications of the findings are analyzed and discussed with respect to consumer health protection.
3 RESULTS & DISCUSSION

3.1 TCC exposure

3.1.1 Investigation of human exposure to TCC after showering


As a basis for a risk assessment of TCC, human exposure levels have to be evaluated. In this study, the urinary concentrations of TCC in human volunteers following exposure by showering with a TCC containing soap were monitored.

A group of six healthy volunteers (age 26-63 years; body weight 60-80 kg) were asked to take a shower with commercially available 0.6% TCC containing soap (Dial Gold soap, Henkel, St. Louis, MO). Aliquots from each urination sample over a time span of 24-48 hours, and a single sample after 72 h were collected. The urine samples were normalized by renal excretion rate based on their creatinine concentration.

The mean soap consumption was 11.7 ± 2.6 g, corresponding to a TCC quantity of 70 ± 15 mg or on average a maximal temporary topical dose of 1 mg/kg body weight. In all samples analyzed TCC and its oxidative metabolites (section 3.3) were barely detectable and only a few samples exceeded the limit of quantification (LOQ, 0.3 nM). Thus, urine concentration of TCC including its oxidative metabolites is negligible, since less than 1 ng/mL is excreted in urine. However, phase II metabolism conjugates were detected in a maximal concentration range of 119 to 1013 nM. The excretion profile of the released TCC over the post exposure time period is absolutely consistent with the semi-quantitative profile of the peak areas for the TCC-\(N\)-glucuronides in all volunteers. These findings are in accordance with earlier reports showing that directly glucuronidated metabolites account for almost all TCC metabolites excreted via the kidneys (Birch et al, 1978; Hiles & Birch, 1978a; Hiles et al, 1978; Scharpf et al, 1975). In all volunteers, maximal TCC excretion
occurred 10-24 hours after exposure by showering and complete clearance required more than 72 h. During repeated daily showering with TCC containing soap, the TCC-N-Gs excretion reached a steady level over time of ~80 µg TCC/g creatinine.

Based on the urine concentration measured, the lowest and highest absorption of TCC following a single exposure could be estimated to be on average 0.5 ± 0.1 mg per shower per person. This corresponds to an absorbed portion of 0.6 ± 0.2% of the TCC amount applied with the soap, which is consistent with earlier findings (Scharpf et al, 1975) that report 0.39% after a similar whole body shower experiment with 14C-TCC containing soap. This good correlation supports the view that the TCC-N-glucuronides levels in urine are highly predictive of the human TCC exposure and TCC-N-Gs levels are ideally suited for monitoring TCC exposure.

Taken together, it is concluded that a small but substantial amount of TCC present in soaps is absorbed by humans through the skin, which in turn could elicit a biological effect (see section 3.4). The excretion rate is slow, and the constant daily use of TCC containing bar soap led to a steady state of TCC levels. However, no evidence for a TCC accumulation in humans was found.
3.1.2 Bioaccumulation, metabolism and excretion of TCC in fish.


Due to its frequent use, levels of TCC up to 5 ppb (16 nM) have been detected in the surface water in the United States (Halden RU, 2004; Halden RU, 2005). The Targeted National Sewage Sludge Survey published in 2009 by the US Environmental Protection Agency (EPA) reported the presence of TCC in all 84 sludge samples analyzed. TCC was found at concentrations of up to 0.44 g/kg, indicating a strong accumulation of TCC in sludge (EPA, 2009). Some studies also showed a bioaccumulation of TCC in aquatic organisms. For most aquatic organisms such as fish, which might enter the human food chain, no data on the bioaccumulation of TCC are available. In order to evaluate if TCC bioconcentrates in fish as it does in other aquatic organisms we exposed 7-day old Japanese Qurt medaka (Oryzias latipes) larvae to 20 ppb TCC and analyzed bioaccumulation, metabolism and excretion.

TCC was rapidly absorbed by medaka, thereby reaching a steady state of 34 ± 2 µmol/kg, corresponding to a log BAF of 2.86 ± 0.05. This BAF is significantly lower than the theoretical log BAF of 3.5 calculated from the n-octanol/water partition coefficient of TCC. This lower bioconcentration can be explained by the rapid metabolism of TCC in medaka fish. All known major oxidative metabolites of TCC (2'-OH-TCC, 3'-OH-TCC, 6-OH-TCC, 3',4' dichloro-4'-hydroxy-carbanilide) and phase II metabolism conjugates thereof were detected in the fish and the medium (see section 3.3). The concentration increased in a time-dependent fashion, thereby reaching a concentration of up to 0.3 nM 2'-O-Gluc-TCC (the glucuronic acid conjugate of 2'-OH-TCC) in the water after 24 hours. Nevertheless, a significant portion (5.5%) of the compound added to the beaker (400 mL) was absorbed by the fish (in total 28 ± 5 mg). In order to evaluate the elimination of TCC, the fish were transferred after 24 h of exposure into clean water. The TCC concentration in fish tissue (40 ± 5 µmol/kg, total amount 1.3 ± 0.2 nmol) rapidly decayed with an initial t\(_{1/2}\) of 1 h and after 48 h only 0.1 % of the initial concentration of TCC (44 ± 2
nmol/kg; total amount 1.5 ± 0.1 pmol) remained in the fish. A minor part (2%) of this rapid elimination is due to the direct diffusion into the water. Most of TCC was eliminated following metabolism. In the first hours after exposure, the concentration of the metabolites 2'-OH-TCC, 3-OH-TCC and 6-OH-TCC, 3,4-dichloro-4'-hydroxyl carbanilide (DHC), 2'-O-SO$_3$-TCC and 2'-O-Gluc-TCC in fish tissue increased and reached the highest levels after 3 h. Thereafter, the levels slowly decreased with a $t_{1/2}$ of about 50 h for 2'-OH-TCC, 3'-OH-TCC and 6-OH-TCC, DHC and 20 h for 2'-O-Gluc-TCC. This time-dependent decline of the concentration in fish tissue can be explained by an excretion of the metabolites in the fish. A simultaneous increase in the concentration of these metabolites in the water was observed.

In summary, metabolism was found to play a major role in the elimination of TCC by fish. Two days after transferring the fish into clean water, the predominant amount (>90%) of TCC was eliminated as metabolites into the water. This elimination phase is surprisingly short for a compound with a $K_{OW}$ of 4.9. For comparison, DDT with an experimentally determined $K_{OW}$ of 4.9-6.9 (Pontolillo & Eganhouse, 2001) showed in a slow rate of elimination in black sea bream (Acanthopagrus schlegeli). In this case only 44% of DDT was eliminated after two weeks (Kwong et al, 2008).

Despite this fast elimination of TCC, our results also showed that TCC accumulates in fish as predicted by the BAF. The resulting high TCC tissue concentration may result in biological effects on fish, since TCC can act as an endocrine disruptor and is an inhibitor of sEH (see chapter 3.4). However, because of the effective metabolism in fish, a trophic accumulation along the food chain seems to be unlikely.
3.2 Determination of TCC in biological samples

3.2.1 Development of a rapid LC-MS method and an immunoassay


and


In order to assess human exposure, analytical methods allowing the quantification of low amounts of TCC and its metabolites in biological samples such as urine and blood are needed. We therefore developed and optimized a LC-ESI-MS/MS method with online-sample preparation by online-solid phase extraction (SPE, Fig. 2) allowing the direct injection of these crude biological samples.

The method allowed baseline separation, selective analysis and quantification of TCC, its metabolites and a few analogues in less than 7 minutes including the online SPE step. The limit of detection (LOD, S/N = 3) for TCC was 0.15 nM (50 pg/mL) equivalent to 6 fmol on column. The method provided a broad linear range of detection over 3 orders of magnitude ($r^2 \geq 0.99$). Using plasma and urine samples spiked with 10, 30 and 100 nM of the analytes, we observed an excellent accuracy for all compounds with a mean recovery rate of 104 ± 8% for both urine and plasma. In addition to the accuracy, the method precision was also excellent with an inter sample variation of less than 5% and an intra sample variation of less than 10% for all analytes. Thus, the direct injection of crude samples after addition of an internal standard and centrifugation in the fully automated ultra-fast online-SPE-LC-MS/MS did not compromise the analytical performance and is ideally suited for the exposure measurement of TCC.
Fig. 2: Scheme of the online-SPE-LC-MS/MS set-up. The sample is transferred onto the SPE column by pump 1 and proteins, salts and other polar matrix compounds are washed to waste (A). After this loading step, the six-port valve is switched (1.0 min), so that the analytes are eluted from the SPE column towards the separation column by pump 2 (B). Following complete transfer, the valve is immediately switched back (2.8 min). The analytes elute, while the SPE column is cleaned and regenerated. In the diagrams the applied gradients (black line) and flow rates (gray line) of the LC-pumps are shown.

A sensitive LC-MS instrument is a very expensive piece of equipment not available in many laboratories. In order to allow the determination of TCC in biological samples with basic laboratory equipment, we developed as a complementary approach an enzyme-linked immunosorbent assay (ELISA) for the analysis of TCC. For this purpose, polyclonal antibodies were used in a competitive assay format.

A key step in the generation of antibodies against small molecules is the synthesis of effective haptens. TCC possesses a low molecular weight and requires conjugation to carrier proteins in order to be immunogenic. Five classes of haptens utilizing a reactive primary amine group or a carboxylic acid group as protein linker were synthesized. The linkers (0, 3 or 4 carbon atoms) were attached either to the 4- or to the 4’-position of the TCC molecule by replacing the chlorine by an isosteric sulfur atom. It turned out that rabbit antibodies generated with a 3-carbon linker at the 4-position bound via a carboxylic acid group to thyroglobulin showed the best
performance. A heterologous ELISA with a competitive hapten containing a piperidine bound to bovine serum albumin (BSA) was then further characterized. The detection range for TCC in buffer was 0.4–11 nM. The assay was selective for TCC, only showing a low cross-reactivity with TCC-related compounds and its major metabolites except for the closely related antimicrobial 3-trifluoromethyl-4,4'-dichlorocarbanilide. In combination with an ethyl acetate liquid-liquid extraction for preparation of biological samples the assay allowed quantification of low part per billion (ppb) levels of TCC. The limits of quantification of TCC were 16 nM in blood/serum and 32 nM in urine. Moreover, the determined levels were similar to those determined by LC-MS. This immunoassay can be used as a rapid and convenient tool to aid researchers monitoring human/environmental exposure to TCC to investigate potential health effects without the need of expensive equipment.
3.2.2 Determination of the best sample matrix for the determination of human TCC exposure


and


The assessment of human exposure requires to carefully choose the best sample matrix and appropriate sample preparation. Urine is the ideal body fluid for exposure assessment, because it can be obtained in a non-invasive manner in large quantities.

We could show that TCC is almost exclusively excreted as N- and N’-glucuronic acid conjugate in human urine. However, no way of synthesizing these metabolites as analytical standards could be worked out by our collaborators and us. Thus, the conjugate amounts have to be determined as TCC after hydrolysis. However, this step has to be carefully optimized, since the commonly used techniques bear the risk of degradation of TCC and its metabolites or incomplete hydrolysis, which would lead to a massive underestimation of the TCC exposure (Fig. 3).

We could show that the hydrolysis with hydrochloric acid (1 M, 100°C, 20 min) as well as a 6-hour treatment with β-glucuronidase (GUS) from *Escherichia coli* are optimal techniques for the release of TCC from its glucuronic acid residues. Interestingly, the most commonly used enzymes for a general conjugate cleavage when determining xenobiotics in body fluids such as GUS from *Helix pomatia* are not suitable for the analysis of TCC exposure based on urine levels (Fig. 3). Apparently, the TCC-N’-glucuronide is not a substrate for the GUS expressed by *Helix pomatia*. 
Fig. 3: Release of TCC from N-glucuronides by enzyme and acid treatment. The progression of the hydrolysis was followed over the incubation period by the peak areas of the two N-glucuronides and the released TCC concentration in the urine of an exposed human. The values for the N-glucuronides were normalized as the percent of control sample at 0 min incubation and set to 100%. The TCC concentration in all diagrams is given as % of the highest measured release of TCC (20 min, acid hydrolysis). The mean and the standard deviation of three injections are presented.

A. Incubation with β-glucuronidase from Helix pomatia type HP-1.
B. Incubation with β-glucuronidase from Helix pomatia type HP-2.
C. Incubation with β-glucuronidase from Escherichia coli.
D. Acid conjugate hydrolysis at 100 °C with 1 M HCl.

Albeit urine analysis provides a good estimate of human exposure to TCC, a calculation of the exact exposure requires the knowledge about the systemic concentration, i.e. the blood levels of TCC. Despite the detection of relevant concentrations in the urine, previous studies failed to detect TCC in human blood samples or reported very low serum levels (about 1.4 nM TCC) in adults living in USA (Ye et al, 2011). This led us to assume that plasma and serum TCC levels do not reflect the actual blood concentration of TCC.
Analysis of spiked blood confirmed the hypothesis that TCC concentrates in the cellular fraction of blood. Ten minutes after incubation of blood with 100 nM TCC the TCC plasma concentration was only 58 ± 6 nM, while the cellular fraction contained 142 ± 4 nM TCC. Furthermore, the ratio between the whole blood and plasma concentrations was 1.9 ± 0.2 and remained stable over the whole incubation period. The same distribution was found in vivo in mice after oral gavage of TCC, the blood/plasma concentration ratio being 1.9 ± 0.2, though the observed TCC whole blood levels among mice varied between 202 ± 7 and 792 ± 34 nM.

Based on these findings, we performed whole blood analysis for the biomonitoring of TCC after exposing human volunteers by a single shower with antibacterial soap (see section 3.1). Whole blood samples were easily obtained from human volunteers by a minimally invasive finger-prick. The obtained blood levels correlated well with the urine excretion data. A maximal blood concentration of 530 nM was determined. The highest urinary excretion of TCC metabolites and a blood level of 285 ± 5 nM were measured in an individual that frequently used TCC containing soap. This clearly demonstrates that measuring the systemic TCC levels is feasible by analyzing whole blood as sample matrix. Several very practical techniques for the sample preparation of whole blood, such as the dried-blood-spot analysis (Li & Tse, 2010) or our approach to mix the whole blood with an excess of water followed by liquid/liquid or solid phase extraction (Schebb et al, 2011b), are nowadays available.

Overall, the two sensitive analytical approaches for the analysis of TCC (i.e. urine analysis after optimized conjugate cleavage and analysis of whole blood) presented in this section allow for the first time to realistically assess the human exposure to TCC.
3.3 Metabolism of TCC

3.3.1 Investigation of oxidative metabolism


Metabolism of TCC by higher organisms such as fish and humans is essential to excrete the compound and prevents bioaccumulation (see section 3.1). Except for the direct conjugation of TCC by UGT, thereby yielding $N$- and $N'$-glucuronides, functional groups have to be integrated into the molecule in phase I metabolism reactions.

In this study, we investigated the oxidative metabolism of TCC in detail. Using microsomal incubations only a very low conversion rate of TCC was obtained, not reflecting the extensive oxidative metabolism in vivo (see section 1). This is most likely related to the poor water solubility of the compound at a concentration of 142 nM (Snyder et al, 2010), thereby leading to a low effective concentration in the assay. In order to allow detailed characterization of possible metabolites, the oxidative metabolism of TCC was mimicked by using an electrochemical cell coupled online to liquid chromatography and electrospray ionization mass spectrometry (EC-LC-ESI-MS). Several studies demonstrated that this electrochemical technique is capable of mimicking the majority of oxidative metabolism reactions, including aromatic hydroxylation, as well as the formation of quinones and quinone imines (Lohmann & Karst, 2008). Coupling EC online to liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS) allows the direct detection of reactive metabolites in the absence of endogenous compounds (Lohmann & Karst, 2007). Moreover, nucleophiles such as GSH or proteins can be added selectively to the online system in order to evaluate the reactivity of a specific metabolite. On the basis of the EC prediction of the TCC metabolism, we thoroughly investigated the CYP mediated metabolism of TCC with liver microsomes from humans (HLM) and rats (RLM).
The electrochemical oxidation of TCC gave rise to ten products. All of these compounds showed a distinct isotopic pattern originating from the natural $^{35}\text{Cl}/^{37}\text{Cl}$ distribution, which indicates the presence of two dichlorinated products and seven trichlorinated products in the LC-MS peaks. Based on high-resolution mass spectrometry, molecular formulas of the products could be calculated. Furthermore, fragment ions, generated by in-source fragmentation in the ESI-interface, have been studied to localize the site of oxidative modification (Fig.4).

**Fig. 4:** Fragmentation behavior of TCC and its products after negative ESI. Fragmentation occurs at both carbon-nitrogen bonds of urea leading to four possible fragments. The m/z and the relative intensities of the fragments are also presented.

Based on these data and the comparison with synthetic reference compounds, three monohydroxylated (3’-OH TCC, 2’-OH TCC and 6-OH TCC) and two dihydroxylated TCC metabolites (2’,6-diOH TCC and 3’,6-diOH TCC) were detected. Moreover, a metabolite, in which a chlorine atom was substituted by an hydroxyl group in the monochloroaniline ring, was detected and identified as 3,4-dichloro-4’-hydroxyl carbanilide (DHC). Hence, the formation of all these metabolites by the CYP enzymes present in rat and human liver microsomes was confirmed.

The metabolites 2’-OH TCC and 6-OH TCC, the dihydroxylated metabolites and DHC can be further oxidized to a quinone imine. Quinones and quinone imines are well-known reactive metabolites, which very often undergo adduct formation with cellular compounds such as glutathione (GSH) and proteins. As a consequence, these compounds can cause toxic effects, especially in case that cellular GSH levels are strongly depleted.
The EC-LC-MS approach indeed led to peaks giving rise to ions with the expected \( m/z \) ratio of the corresponding quinone imines (TCC+O-2H, TCC+2O-2H, TCC+O-Cl-2H). In order to investigate if the quinone imines formed by oxidative metabolism of TCC are reactive, the EC effluent was mixed with glutathione and the model protein \( \beta \)-lactoglobulin A. The quinone imines of 2'-OH TCC and 6-OH TCC and DHC gave rise to both GSH and GSH adducts. The formed adducts were thoroughly characterized by means of HR-MS as shown in Fig. 5 for the formation of DHC and 2-OH-TCC quinone imines.

![Mass spectra and deconvoluted mass spectra](image)

**Fig. 5:** Mass spectra and deconvoluted mass spectra of (a) \( \beta \)-lactoglobulin A (\( \beta \)-LGA), (b) \( \beta \)-LGA after reaction with electrochemically oxidized TCC and (c) \( \beta \)-LGA after reaction with electrochemically oxidized 6-OH TCC. The mass differences are in agreement with the calculated differences between the average masses of covalent adducts between (b) \( \beta \)-LGA and DHC quionone imine and (c) \( \beta \)-LGA and 6-OH TCC quinone imine. The same mass difference as for 6-OH TCC was observed when mixing oxidized 2-OH TCC and \( \beta \)-LGA (data not shown).
In summary, this study led to the detection of novel metabolites of TCC. A new, dechlorinated hydroxylated TCC metabolite as well as reactive quinone imine species, which tend to bind to proteins and GSH, were described. These findings may explain the previously reported observation of unidentified polar TCC metabolites and protein binding *in vivo*. However, neither microsomal studies nor the data obtained by the EC-based metabolism simulation can completely depict processes taking place *in vivo*. The fact that the metabolization of TCC to reactive products has been identified in an *in vitro* system and that it may lead to the formation of GSH adduct does not necessarily imply that they are formed in cells and lead to toxicity in them.
3.3.2 Protein adduct formation in skin cells


In this study we investigated if TCC is metabolized in skin, the primary tissue exposed to a personal care product ingredient. Since keratinocytes in the epidermis and in culture express substantial phase I and phase II activities, this study particularly explores the hypothesis that, upon dermal exposure, TCC undergoes biotransformation, which leads to the formation of potentially deleterious reactive metabolites (see section 3.3.1).

Spontaneously immortalized keratinocytes (SIK), which have been successfully used to demonstrate the importance of CYP mediated toxic effects on skin in the past, were used as an in vitro skin model. Following incubation of SIK with TCC, the compound was rapidly absorbed by the cells. A small portion of the absorbed TCC was oxidatively metabolized to 2´OH-TCC, 3´OH- 6-OH-TCC, DHC and their conjugates. Preincubation with 10 nM TCDD - a strong Ah-receptor agonist - dramatically increased the metabolism of TCC in the keratinocytes, and the relative conversion (cellular metabolite concentration versus cellular TCC concentration) increased from ≈ 0.5% to 15%. In contrast to the metabolite patterns in blood, bile and urine, the amount of unconjugated oxidative metabolites of TCC exceeded by far the level of conjugated (phase II) species.

Further oxidation of oxidative metabolites can yield reactive quinone imines directly binding to cellular proteins (see section 3.3.1). Protein adducts were determined following incubation of SIK with $^{14}$C labeled TCC and accelerator mass spectrometry analysis. In the presence of TCDD, up to 23 ± 2 pmol TCC adducts/mg protein were detected after a 24 h incubation, and the protein adducts increased in a time-dependent manner. In the absence of TCDD a very low number of adducts (about 1 pmol/mg protein), slightly above the background signal, was detected, which is consistent with the low metabolic conversion of TCC in SIK.
These results clearly demonstrate that oxidative TCC metabolism leads to reactive intermediates in human cells. These metabolites, presumably quinone imines, can covalently bind to proteins, which was clearly shown upon induction of drug metabolizing enzymes by TCDD.

Regarding toxicity it has to be kept in mind that the reactivity of a chemical to produce a complete antigen by covalently adducting a carrier protein is the major factor leading to allergic skin sensitization. In the absence of CYP induction, the low level of TCC protein adducts suggests that the probability of inducing adverse effects in skin cells is low. However, the substantial level of protein adducts after TCDD treatment raises the possibility of skin sensitization after exposure to Ah receptor agonists. TCC itself does not act as an Ah receptor agonist (Morisseau et al, 2009). However, Ah receptor agonists are widely encountered in tobacco smoke, pharmaceuticals, food constituents and other consumer products. Thus, this study indicated the possibility that the combined exposure of TCC and Ah-receptor agonists might contribute to the development of allergic contact dermatitis in humans.
3.3.3 Activation of TCC by CYP1A1 yielding glutathione adducts


In an electrochemistry liquid chromatography mass spectrometry (EC-LC-MS) approach we could show that further oxidation of the main oxidative products of TCC, namely 2’OH-TCC, 6’OH-TCC and DHC, can generate reactive quinone imines (see section 3.3.1). Accordingly, the incubation of immortalized keratinocytes with TCC led to small amounts of TCC covalently bound to cellular macromolecules (see section 3.3.2). In these experiments adduct formation and oxidative metabolism of TCC was enhanced by pre-incubation with the Ah receptor agonist TCDD, thus indicating a role for inducible CYP isoforms in reactive metabolite formation in keratinocytes.

Therefore, in the present study we studied whether TCC is activated by the main TCDD-inducible CYP isoforms CYP1A1 and CYP1B1. Moreover, the structure of the reactive metabolites and the route of metabolic activation were characterized based on HRMS analysis of the resulting GSH adduct. To obtain quantitative information on GSH adduct formation, incubations with 14C-TCC, GSH and purified enzymes followed by LC analysis were carried out. The radioactivity eluting from the HPLC column was monitored by scintillation counting.

Incubations with CYP1A1 gave rise to a significant peak in the radiochromatogram, containing 500 pmol product equivalent to 1% of the initial TCC amount, while no radioactive peak was observed in that retention time window in the case of CYP1B1 incubations. LC-MS analysis of extracts prepared from incubations containing CYP1B1 showed no evidence of reactive metabolite formation. In HRMS analysis, the CYP1A1 metabolite showed an isotopic pattern of a compound bearing two chlorine atoms. This suggests a loss of one chlorine by oxidative metabolism, as described for the formation of DHC (see section 3.3.1). The exact (monoisotopic) mass of this metabolite was determined to be 601.0801 Da, which exactly
corresponds to the calculated mass of a DHC-GSH adduct. Moreover, the observed fragmentation pattern is consistent with the suggested structure of a DHC-GSH adduct (Fig.6)

![Chemical structures](image)

**Fig. 6:** Fragmentation pattern of TCC-GSH adduct: **Left** Suggested structure of the adduct; dashed lines depict suggested sites of fragmentation (a-f). Adjacent numbers are the m/z ratio of the resulting fragments. **Right:** Suggested structures of fragments.

The DHC-GSH conjugate can be formed from TCC by dehalogenation and hydroxylation followed by nucleophilic addition of GSH to the monochloraniline ring. This was confirmed by incubation of CYP1A1 with DHC as precursor. This incubation led to a peak at the same retention time showing the same ions in ESI-MS, ESI-MS/MS as the incubation of TCC. Thus, it can be concluded that TCC is metabolically activated by oxidative dehalogenation at the monochloraniline ring and hydroxylation to a p-quionone imine, which generates the glutathione adduct.

Our studies using both LC-MS and LC radioprofile analysis clearly demonstrated GSH adduct formation following oxidative conversion of TCC by CYP1A1. However, it is difficult to extrapolate from the observed formation rate *in vitro* to the generation of metabolites in cells or whole tissues *in vivo*.

The metabolism of TCC to both oxidative metabolites and *N*-glucuronides is substantially underestimated by *in vitro* microsomal incubations (see section 3.3.1
and 3.3.4). Thus, TCC activation might occur to a much higher extent \textit{in vivo}. In fact, the suggested precursor of the reactive metabolite has been found in a number of different organisms such as rat, mice or medaka fish (see section 3.1.2).

The finding that TCC-GSH adduct and thus reactive metabolite formation is catalyzed by CYP1A1 is consistent with the data showing that TCC protein adduct formation in keratinocytes is substantially increased following induction with the Ah receptor agonist TCCD (see section 3.3.2).

The DHC-GSH adduct was also found in incubations of SIK cells with TCC. Although this peak represents only a small portion of the metabolites generated (about 1% of the amount of TCC added to the cells), it is qualitatively and quantitatively consistent with the formation of small amounts of protein-bound metabolite and demonstrates the ability of keratinocytes to inactivate the electrophile through conjugation with GSH.

The biological consequences of reactive metabolite formation from TCC cannot be assessed with certainty, but the slow rates of metabolism to both protein- and glutathione-reactive intermediates would suggest minor if any toxic effects. One concern is whether these reactive metabolites bind to nucleophilic sites in DNA.

The quinone imine intermediates generated from TCC are soft Lewis acids and would be less likely to react with the hard nucleophilic sites (strong Lewis bases) in DNA bases. In order to substantiate this assumption, a preliminary mutagenicity study of TCC in TCDD-induced keratinocytes based on a modified hypoxanthine-guanine phosphoribosyltransferase (HPRT) forward mutation assay was carried out. Mutagenicity was determined by determining the number of colonies formed. As shown in Table 1 mutated colonies were below the limit of detection in the case of TCC while the positive control, the aromatic amine o-aminoazotoluene (AAT), clearly elicited mutations.
**Table 1:** Lack of Mutagenicity of TCC

<table>
<thead>
<tr>
<th>Agent</th>
<th>Conc. (µM)</th>
<th>Mut/Surv</th>
<th>Freq x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT</td>
<td>13</td>
<td>24/273,000</td>
<td>88</td>
</tr>
<tr>
<td>TCC</td>
<td>4</td>
<td>0/375,000</td>
<td>&lt; 3</td>
</tr>
</tbody>
</table>

SIK cultures were treated with o-aminoazotoluene (AAT) and triclocarban (TCC) at the indicated concentrations (Conc). Total surviving colonies before selection (Surv) were estimated from colony forming efficiencies measured in the absence of selection. The frequency (Freq) of mutant colonies (Mut) is given per million survivors.

Overall, the formation of reactive metabolites, particularly at the levels described here and in earlier protein adduct studies does not result in a high level of concern for adverse health effects when using TCC in personal care products. Given the high production volume of TCC and the widespread human exposure, it is surprising that the formation of reactive TCC metabolites and TCC-GSH adducts has not been reported during the last 50 years of use of the compound.
3.4 Investigation of glucuronidation of TCC and its metabolites


A significant portion of TCC in soaps is percutaneously absorbed by humans during and after showering (see section 3.1.1). Moreover, it has to be assumed that TCC from contaminated drinking water or food will be extensively absorbed because TCC shows a high bioavailability after oral dosing.

In mammals, TCC is rapidly metabolized. The main metabolite detected in human urine results from direct conjugation, presumably *N*-glucuronidation of TCC. The majority of the absorbed TCC is metabolized by cytochrome P450 enzymes (CYP) to three hydroxylated TCC species, namely 2′-OH-TCC, 3′-OH-TCC and 6-OH-TCC, and DHC with the ortho-hydroxylated species, 2′-OH-TCC and 6-OH-TCC being the main metabolites (see section 3.3.1). All metabolites undergo extensive phase II metabolism and the glucuronic acid conjugates of the hydroxylated TCC species account for the majority of TCC metabolites in the mammalian bile (Birch et al, 1978).

Thus, conjugation with glucuronic acid plays a key role in the renal as well as biliary elimination of TCC. However, no information regarding the role of UDP-glucuronosyltransferases (UGTs) in the conjugation of TCC and its metabolites was available. Therefore, the aim of this study was to identify the UGTs involved in the conjugation of TCC and its oxidative metabolites as well as the biochemistry and kinetics of the UGT-catalyzed reactions based on microsomal incubations from humans, monkeys, rats and mice.

The hepatic UGTs 1A1, 1A3 and 1A9 showed high activities regarding the conjugation of all hydroxylated metabolites. The highest activity was found in the case of UGT1A9, which favoured 6-OH-TCC as substrate followed by 2′-OH-TCC and 3′-OH-TCC. As expected from these results, HLM showed a high conjugating activity, the conversion rate order being 6-OH-TCC > 3′-OH-TCC > 2′-OH-TCC.
Rapid conjugation of the oxidative TCC metabolites was also found in liver microsomes of mouse, monkey and rat, thus indicating that mammalian liver UGTs generally show a high affinity for hydroxylated TCC metabolites. These results mechanistically explain why the glucuronides of hydroxylated TCC species are by far the dominating metabolites found in mammalian bile. Our activity screening also unveiled a very high conjugation activity of the extrahepatic UGTs 1A7 and 1A8.

It is concluded that oxidative TCC metabolites will also be rapidly conjugated in extrahepatic tissues. This assumption is supported by the analysis of kidney and intestine microsomes, which conjugated oxidative TCC metabolites at rates comparable to the liver microsomes. The substrate selectivity pattern of kidney was 6-OH-TCC > 2'-OH-TCC > 3 OH-TCC, in line with the high expression of UGT1A9 in the kidney. Similarly, intestine microsomes conjugated 3'-OH-TCC the fastest, followed by 2'-OH-TCC and 6-OH-TCC, which is identical to the pattern observed for the intestinal UGT1A8. All tested microsomes showed a low $K_M$ and a high $v_{max}$, resulting in a high apparent intrinsic clearance for phase I metabolites of TCC. In all four species investigated, the conjugation of 2'-OH-TCC followed Michaelis-Menten-type kinetics, as indicated by a linear correlation in the Lineweaver-Burk and Eadie-Hofstee plots (Fig.7).
circulation will probably occur in the case of biliary excreted TCC metabolites. The clearance of 2'-OH-TCC by MLM and RLM was comparable or higher than the reported values for reference UGT substrates such as mice, whereas no species specificity, TCC-N-glucuronides were detected after incubation with HLM, CLM and MLM, but not after incubation with RLM. However, none of the microsomal incubations revealed the formation of TCC-N-glucuronides at a rate higher than 1%.

\[ v_{\text{max}} = 671 \text{ nM/min} \]

\[ K_M = 1.4 \text{ µM} \]

\[ v = -0.7x + 473 \]

\[ y = 0.00417 + 0.00298 \]

\[ 1/v = K_M + 1/v_{\text{max}} \]

\[ y = 0.00149x + 0.00208 \]

The clearance of 2'-OH-TCC by MLM and RLM was comparable or higher than the reported values for reference UGT substrates such as para-nitrophenol, propofol and mycophenolic acid. However, since both N- as well as O-glucuronides can be cleaved by β-glucuronidase from Escherichia coli (see section 3.2.2) enterohepatic circulation will probably occur in the case of biliary excreted TCC metabolites.

N- and N'-glucuronides are the major urinary TCC metabolites in monkeys and in mice, whereas no N-glucuronidation was observed in rats. In accordance with this species specificity, TCC-N-glucuronides were detected after incubation with HLM, CLM and MLM, but not after incubation with RLM. However, none of the microsomal incubations revealed the formation of TCC-N-glucuronides at a rate higher than 1%.
As already discussed, microsomal incubations underestimate TCC metabolism because of its poor solubility. Nevertheless, the LC-MS based activity screening showed that UGT1A9 gave rise to significant, although low levels of $N$-glucuronides and allowed a tentative identification of UGT1A9 as the only UGT catalyzing the $N$-glucuronidation of TCC. The identification of this isoform is also consistent with the species specificity of TCC-$N$-glucuronidation. While human, monkey, and mouse express an active enzyme, UGT1A9 is a pseudogene in rat (Mackenzie et al, 2005). Consequently, only incubation with RLM did not lead to any $N$-glucuronides in our experiments. Moreover, the conjugation of the urea nitrogen in TCC is in line with the substrate specificity of UGT1A9.

In summary, our study showed that all major oxidative metabolites of TCC are rapidly conjugated with glucuronic acid by microsomes from liver, kidney and intestine. A broad variety of UGTs show a high affinity for the hydroxylated TCC metabolites, the activities of UGT1A7, UGT1A8 and UGT1A9 towards the oxidative metabolites of TCC being particularly high.

In contrast, hardly any $N$-glucuronides of TCC are formed in microsomal incubations. Nevertheless, based on the sensitive LC-ESI-MS detection of low amounts of the formed product, UGT1A9 could be tentatively identified as a major UGT in the metabolization of TCC.
3.4 Off target effects of TCC on mammals

3.4.1 Investigation of inhibition of mammalian enzymes by TCC


and


Pharmacological or toxicological actions of a compound in a living system are generally mediated by the interaction with a receptor or enzymatic pathway, with the exception of the modification of cellular macromolecules.

In previous sections we could show that a significant portion of TCC in soaps is percutaneously absorbed by humans during and after showering. The absorbed portion is systemically available and intensively metabolized.

In order to evaluate if TCC interferes with metabolic enzymes and thus modulates mammalian organisms, the compound and its metabolites were screened for the potential inhibition of a small library of human enzymes: human esterases (hCE), 1-3 fatty acid amide hydrolase (FAAH), CYP1A1, glutathione-S-transferases (GSTs) and mitochondrial epoxide hydrolase (mEH). As shown in Table 2 TCC and/or its metabolites either did not affect or moderately inhibited the activity of the above-mentioned enzymes. Thus, it is unlikely that TCC could modulate the activities of these enzymes \textit{in vivo} and cause significant biological effects.
Table 2: Screening of TCC and its metabolites for non-intended effects on the activity of human enzymes.

<table>
<thead>
<tr>
<th></th>
<th>hCE1</th>
<th>hCE2</th>
<th>hCE3</th>
<th>FAAH</th>
<th>CYP1A1</th>
<th>GSTs</th>
<th>mEH</th>
</tr>
</thead>
<tbody>
<tr>
<td>test conc.(µM)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>(%) inhibition</td>
<td>13 ± 6</td>
<td>12 ± 4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>13 ± 4</td>
<td>&lt;1</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>TCC</td>
<td>22 ± 6</td>
<td>11 ± 5</td>
<td>&lt;1</td>
<td>3.5 ± 0.4</td>
<td>3 ± 3</td>
<td>3.5 ± 0.4</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>2'OH-TCC</td>
<td>13 ± 4</td>
<td>8 ± 1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>32 ± 2</td>
<td>&lt;1</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>2'SO3-O-TCC</td>
<td>+6</td>
<td>12 ± 4</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>13 ± 4</td>
<td>&lt;1</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

When TCC was tested in a high-throughput assay by using a fluorescent substrate an IC50 value of 39 ± 3 nM for the inhibition of the soluble epoxide hydrolase (sEH) was obtained. The inhibition of sEH by TCC was again tested in a new LC-MS-based assay using the endogenous substrate (see section 3.4.2). An IC50 of 24± 5 nM was obtained, thus confirming that TCC is a strong sEH inhibitor.

All TCC metabolites (see section 3.3) show a lower inhibition potency (higher IC50 values) than the parent compound. In particular the main plasma metabolite 2’SO3-O-TCC bearing a bulky sulphate group at the ortho-position to the urea moiety reduces the inhibitory activity towards sEH about 100-fold (Fig.8). Thus, the metabolism of TCC leads to a biological deactivation of TCC with respect to sEH inhibition. The in vitro potency of TCC-N-Gs was not determined, since reference compounds were not available. However, based on the extensive structure-activity relationship of sEH inhibitors it is highly unlikely that these major urinary metabolites would inhibit sEH (Morisseau et al, 1998; Morisseau et al, 1999; Rose et al, 2010). We predict that the glucuronic acid group would prevent hydrogen bonding of the urea moiety at the active site of the enzyme and based on our crystal structure data is too large to fit in the catalytic pocket.
With a nanomolar IC$_{50}$, TCC may inhibit sEH at human exposure-relevant concentrations. In order to evaluate if exposure to TCC in personal care products could lead to a systemic sEH inhibition, we administered topically 1.5 mg TCC/kg body weight (a dose in the range of human exposure) and a higher dose of 4 mgTCC/kg body weight to rats. Plasma levels of epoxy fatty acids and their corresponding sHE-generated degradation products, the dihydroxy fatty acids from arachidonic acid and other polyunsaturated fatty acids, were quantified as biomarkers of sEH activity in plasma. Significant changes in the ratio of epoxy/dihydroxy fatty acids were not observed in either the low dose or the high dose groups. Since the ratio of epoxy to dihydroxy fatty acids reflects systemic inhibition of she, it is concluded that these topical TCC doses did not significantly alter systemic sEH activity.
3.4.2 Development of methods to study the inhibition of the soluble epoxide hydrolase


and


In these studies new high-content screening tools to study the inhibition of sEH, the potentially primary target of TCC in mammals, were developed: a LC-MS based-assay using the endogenous substrate and low concentration of enzyme as well as a liquid chromatography-biochemical detection assay allowing to determine the potency of individual compounds in mixtures. Several in vitro assays have been described for the determination of sEH inhibition utilizing surrogate substrates such as cyano(6-methoxy-naphthalen-2-yl)methyl trans-[(3-phenyloxiran-2-yl)methyl] carbonate (CMNPC) or tritium-labelled trans-diphenyl-propene oxide (t-DPPO) (Morisseau & Hammock, 2007). However, due to the distinct recognition of the dissimilar substrates by the enzyme, the measured potencies of sEH inhibitors (sEHIIs) may differ between these methods. In order to obtain results predictive of the in vivo potency of the compounds, inhibition assays utilizing the natural substrates are advantageous.

Known natural substrates of sEH are epoxy fatty acids, which are metabolized to their corresponding fatty acid diols. Among the epoxy fatty acids, arachidonic acid epoxides (EpETrEs) are the best characterized.

In a first step, we developed a rapid LC-MS readout together with a streamlined sEH inhibition assay in microtiter plate format utilizing the endogenous substrate 14(15)EpETrE. Incubation samples were directly injected without purification into an online-solid phase extraction (SPE) liquid chromatography electrospray ionization
tandem mass spectrometry (LC-ESI-MS/MS) set-up allowing a total run time of only 108 s for a full gradient separation.

Analytes were extracted from the matrix within 30 s by turbulent flow chromatography. Subsequently, a full gradient separation was carried out and analytes were detected with high sensitivity by ESI-MS/MS in SRM mode. The substrate 14(15)-EpETrE eluted at a stable retention time of 96 ± 1 s and its sEH hydrolysis product 14,15-DiHETrE at 63 ± 1 s with narrow peak width. The method displayed excellent analytical performance, a low limit of detection, a linear range of over three orders of magnitude and a negligible carry-over.

The enzyme assay was carried out in a 96-well microtiter plate format with a substrate concentration at its $K_m$-value, a low enzyme concentration (33 pM) and an optimal salt and protein concentration. Extremely reproducible sigmoidal dose-response curves of 12 concentrations for each inhibitor were obtained in only 22 min allowing the precise determination of IC$_{50}$ values. Because of the low enzyme concentration, this method allows the quantitative evaluation of potent sEHIs showing picomolar potencies because only 33 pM sEH were included in the reaction vessel. This was demonstrated by ranking 10 compounds according to their activity, all of them yielding an IC$_{50}$ ≤ 1 nM in the fluorescence method.

The comparison of 13 inhibitors having IC$_{50}$ values higher than 1 nM showed a very good correlation with the fluorescence method (linear correlation coefficient 0.9, slope 0.95, Spearman’s rho 0.9). However, for individual compounds up to 8-fold differences in potencies between the enzyme-based and the fluorescent method were obtained. Therefore, it is concluded that enzyme assays using natural substrates are indispensable for a reliable structure-activity relationship determination of sEH inhibition.

Despite the advantages of the developed MS-based assay microtiter plate assay format, it fails to evaluate sEHI potencies in mixtures, e.g. those obtained from microsomal incubations (see section 3.3). Metabolites can be active towards the
same pharmacological target or against an off-target. Screening metabolic mixtures for individual bioactive metabolites is not possible with standard endpoint plate reader-based screening methodologies. In order to assess the bioactivity of individual metabolites, fractionation processes have to precede the screening. The other possibility is to synthesize the metabolites as carried out for TCC in section 3.3. However, fractionation processes and organic synthesis followed by purification are time-consuming and costly. One way of assaying individual potencies of inhibitors in mixtures is the application of an on-line post column screening approach known as high resolution LC with on-line biochemical detection (BCD) (Schebb et al, 2008). In a second step we developed a LC-BCD method for the analysis of sEHIs. In this case the enzyme assay is carried out as a continuous-flow-assay in the eluate of the LC (Fig. 9). The steady-state concentration of the product formed by the enzyme is monitored by a fluorescence measurement and serves as baseline for the detection. Eluting inhibitors are detected as negative peaks in this signal and the active compounds are directly characterized/identified by simultaneous UV-ESI-MS detection. For the assay, the non-fluorescent substrate (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester (PHOME) is used, which is converted to the fluorescent product 6-methoxy-2-naphthaldehyde in combination with purified recombinant human sEH.
The performance of the assay was investigated by analyzing the potency of several known inhibitors of different potencies (100 fold difference in IC$_{50}$). We could show that the concentration-response curves obtained based on the peak height overall led to the same potency as microtiter plate format assays. Moreover, the LC-BCD method allowed to detect both active and inactive metabolites sEH inhibitors in crude mixtures obtained from microsomal incubations.

Given the increasing interest in sEH as drug target in the case of various diseases, the new high-content screening techniques described in this section could be important tools for drug development. The LC-MS based assay allows the precise characterization of the potency of sEHIs, indispensable for the ranking of compounds by their in vitro potency. The LC-BCD method may pave the road for the detection of new classes of sEHIs in natural products or crude mixtures arising from organic synthesis. Moreover, metabolism studies with LC–BCD as read-out will allow
the identification of active metabolites in early stages of lead development and thus assist in the identification of the best compounds as drug candidates.
4 Comprehensive Discussion

The present work aimed to gain data to perform a risk assessment of the use of TCC as antibacterial in PCP such as cosmetics. Human exposure, bioconcentration in fish, metabolism and off target effects of TCC on mammalian enzymes were investigated.

In a first step sensitive and robust methods were developed as tools for the analysis of human and environmental exposure to TCC (see section 3.3.1). LC-ESI-MS methods for TCC have been developed before and applied to environmental samples like water and sludge (Cha & Cupples, 2009; Garcia-Ac et al, 2009; Gonzalez-Marino et al, 2009; Halden & Paull, 2004; Sapkota et al, 2007). However, these methods do not include the quantification of metabolites and their sample preparation strategies were not suited for investigating biological samples, e.g. plasma and urine. Therefore, we developed an LC-ESI-MS method with automated sample preparation by online solid phase extraction (SPE). This approach has successfully been demonstrated for the detection of TCC among other contaminants in breast milk (Ye et al, 2006). In comparison with this method the newly developed method was significantly faster (7 minutes vs. 24 minutes). Moreover, all major metabolites of TCC were simultaneously detected. In fact, the slightly modified method - including metabolites characterized and synthesized within this project - published by (Schebb et al, 2011a) is the most comprehensive method for the detection of TCC and its metabolites that has been described up to now. It should be noted that the automated sample preparation did not negatively influence the sensitivity of the method. With a high chromatographic resolution, an excellent signal to noise ratio (s/n) was observed for the peaks of TCC and its metabolites, thereby yielding a lowest detection limit of 0.3 pM (30 amol on column). This is better or comparable to all other methods described so far for the quantification of TCC (Cha & Cupples, 2009; Garcia-Ac et al, 2009; Gonzalez-Marino et al, 2009; Halden & Paull, 2004; Sapkota et al, 2007).
A major factor allowing this sensitivity is the used state-of-the-art triple quadrupole MS instrument with efficient ion optics and powerful turbo-pumps yielding an excellent vacuum. This is an expensive equipment with high running and maintenance costs. As a consequence, this kind of LC-MS instruments is not available in all laboratories.

In order to allow TCC exposure monitoring with basic laboratory equipment, a competitive indirect enzyme-linked immunosorbent assay using polyclonal antibodies from rabbit was developed. Based on the synthesis of optimized haptens, this method demonstrated to be very sensitive and, most importantly, specific for TCC. Among the analogues tested, only the closely related antimicrobial 3-trifluoromethyl-4,4’-dichlorocarbanilide showed cross-reactivity. In combination with optimized sample preparation strategies for urine and blood (see section 3.2.2), this method made the quantification of trace levels of TCC and its metabolites in samples from exposed humans and animals feasible. Having these two independent detection methods at hand significantly improved the quality of the analytical data allowing to confirm unexpected findings such as high renal TCC excretion levels or low plasma concentrations.

The application of these methods to the investigation of human exposure by showering with a soap containing 0.6% TCC allowed demonstrating that the urine of the exposed subjects contained high concentrations of TCC metabolites of up to 1 µM. It is noteworthy that these levels are consistently higher than those reported from previous showering or bathing studies with TCC containing soaps. Specifically, Gruenke et al. (1987) reported 30 ng/mL TCC equivalent (60 nM) in the urine of users of TCC containing bar soap. Another study even did not detect TCC in the urine of human subjects after intensive 28-day bathing with 2% TCC containing soap(Howes & Black, 1976), which is most likely due to a lack of appropriate conjugate hydrolysis.
We could further demonstrate for the first time that renal excretion of TCC conjugates varies strongly among individual subjects (up to 10-fold). These differences in absorption and metabolism should be taken into account when evaluating a potential impact on human health in a larger population (see below).

The total clearance of TCC in urine following a single exposure required approximately 72 hr. In spite of this slow elimination, no accumulation of TCC or its metabolites was detected during a period of two weeks of daily showering with TCC containing soap. However, urinary excretion reached a constant level of 123 ± 31 µg TCC-glucuronide/g creatinine, thus indicating a steady state of TCC body burden.

When investigating bioconcentration of TCC from water in fish using Medaka larvae as model organism, we found 700-fold higher levels in fish tissue compared to the water concentration (log BAF 2.86). This is significantly lower than the bioconcentration predicted from the $K_{OW}$ of TCC. Moreover, we observed a rapid elimination of TCC after transferring the fish to fresh water. Together with the detection of oxidative as well as phase II metabolites of TCC in the water this finding clearly indicates that fish are able to efficiently metabolize and excrete absorbed TCC. A trophic accumulation along the food chain of TCC is therefore unlikely.

Overall our exposure studies indicate that TCC does not accumulate in higher organisms such as mammals and fish possessing both CYP enzymes for oxidative phase I metabolism and UDP-glucuronosyltransferases (UGTs) for phase II conjugation.

It has been suggested that the levels of compounds found in sewage sludge are good predictors for compounds representing a health risk (Venkatesan & Halden, 2014). However, in the case of TCC, the most abundant synthetic compound in sludge, we do conclude that higher organisms can efficiently handle the lipophilic compound, i.e. excrete it swiftly due to metabolism. Nevertheless, this does not automatically mean that the compound is safe, since our studies also show that a
relevant portion of TCC is dermally absorbed by humans, thereby becoming systemically available (blood levels of up to 500 nM) upon a single exposure to TCC. Consequently, a chronic TCC exposure causes a relevant body burden of the compound (blood concentration ~200 nM). For aquatic organisms chronic exposure can be particularly problematic, since the mean tissue concentration is about 700-fold higher than that of the surrounding water, which cannot be modulated by rapid metabolism.

Metabolism plays a pivotal role in the pharmacokinetics/toxicokinetics of TCC. Therefore, we investigated in detail the oxidative metabolism by using an electrochemistry approach, classical microsomal incubations and in vivo experiments in rodents. Regarding the oxidative metabolism of TCC, our findings largely support the findings reported in the 1980’s (Birch et al, 1978; Gruenke et al, 1987; Hiles & Birch, 1978a). The main metabolites are formed by aromatic hydroxylation in the 2’, 3’ and 6 position (Fig. 10).

![Fig. 10 Overview of TCC metabolism in humans](image-url)
No evidence for an oxidation of the urea nitrogens or a cleavage of the urea moiety to potentially toxic aniline derivatives, which can be activated to nitrosamines, was found. 2'-OH-TCC and 2'-OH-TCC conjugates were identified as the main metabolites detected in human urine, fish as well as plasma and urine of mice and rats.

Our studies led to the identification of a new oxidative TCC metabolite, 3,4-dichloro-4'-hydroxy-carbanilide (DHC), formed by an uncommon CYP catalyzed aromatic dehalogenation and hydroxylation. This compound was synthesized and confirmed to be a metabolite being formed in significant concentrations e.g. in fish or skin cells. Moreover, our studies demonstrated for the first time that the o-hydroxylated metabolites 2'-OH-TCC and 6-OH-TCC as well as DHC can be oxidized to reactive quinone imines. Formation of reactive metabolites is a major mode of action, which can lead to severe toxic effects. Detailed investigation of reactive metabolites of TCC indicated that the metabolic activation occurs predominantly via conversion to DHC and oxidation to a \( \rho \)-quinone imine, which forms glutathione adducts (Fig. 11).

![Fig. 11 Metabolic activation of TCC via dehalogenation and hydroxylation to DHC by CYP1A1 and subsequent oxidation to a \( \rho \)-quinone imine.](image)

We were able to identify the inducible CYP1A1 as a CYP isoform catalyzing the metabolic activation of TCC. Consequently, Ah-receptor agonist TCCD massively increased DHC formation and covalent protein modification in a keratinocyte cell culture. This gives reason to believe that TCC could be activated to electrophilic reactive metabolites in skin in case of co-exposure with Ah-receptor agonists such as e.g. polycyclic aromatic hydrocarbons.
The reactivity of a chemical to produce a complete antigen by covalently adducting a carrier protein is the major factor in inducing allergic skin sensitization (Roberts & Aptula, 2008). In the absence of CYP induction, the low level of TCC-protein adducts suggests that the probability that TCC may lead to adverse effects in keratinocytes is low. This is in line with the safety evaluation of TCC by the Scientific Committee on Consumer Products of the European Commission describing no effect of the compound in skin sensitization assays (EuropeanUnion, 2005).

Nonetheless, upon co-exposure with Ah-receptor agonists, which are found in a large number of household products, the situation might be different. Our data shows substantial – but still low - levels of protein adducts in skin cells after CYP1A1 induction after TCDD treatment, which raises the possibility of skin sensitization under special circumstances. The unrecognized possibility that TCC might contribute to the development of allergic contact dermatitis is a surprising key finding of our studies, which merits further investigation in the context of consumer health protection.

After absorption and initial oxidation of TCC, conjugation reactions of the hydroxylated metabolites compete with further oxidation reactions leading to the reactive quinone imines. To obtain quantitative information on the clearance of potential precursors of reactive metabolites, we investigated in detail the glucuronidation of the phase I metabolites of TCC. It turned out that the hydroxylated TCC derivatives were rapidly conjugated by microsomes from liver, kidney and intestine. A broad variety of UGTs possess a high affinity for the hydroxylated TCC metabolites, UGT1A7, UGT1A8 and UGT1A9 showing high activities. These findings indicate that those metabolites are efficiently glucuronidated upon their formation in various human tissues.

Following exposure, we found high levels of TCC in hydrolysed urine samples of men and mice but not in the rat. This confirmed earlier findings that renal elimination of TCC mainly occurs via direct phase II metabolism (Birch et al, 1978). These
metabolites were thoroughly characterized by means of LC-MS and tentatively identified as TCC-N-Gluc and TCC-N'-Gluc (Fig 10).

When investigating the UGTs responsible for this conjugation, we could identify UGT1A9 as the isoform showing a significant activity regarding the direct conjugation of TCC despite the low conversion rate of TCC in cell-free assays. This tentative identification of UGT1A9 being the key enzyme in TCC-N-glucuronidation provides a convincing explanation for the observed species selectivity of this metabolic pathway. While humans and mice express an active enzyme, UGT1A9 is a pseudogene in the rat (Mackenzie et al, 2005). Moreover, the determined activity of the microsomal fractions of different tissues supported the above-mentioned view, since UGT1A9 activity was only found in liver, intestine and kidney, which is in accordance with the expression pattern of UGT1A9 (Ohno & Nakajin, 2009).

Overall, the observed intense mammalian metabolism of TCC is consistent with the effective elimination of the compound in vivo. For the direct conjugation and elimination via the urine as well as the oxidative metabolism, conjugation and elimination via the bile, glucuronidation plays a pivotal role in TCC metabolism and excretion. For the safety evaluation of the compound, it should be taken into account that several genetic diseases are associated with a reduced UGT activity such as the Gilbert’s syndrome, which affects up to 12% of the population (Rudenski & Halsall, 1998), or the Crigler-Najjar syndrome. These genetic disorders directly affect several members of the UGT1 enzyme family because of common promoter regions and identical exons (Tukey & Strassburg, 2000). Thus, these syndromes would directly impair excretion of TCC, despite the broad UGT conjugating activity spectrum. Hence, the conclusion in the case of consumer safety is similar to that regarding the formation of reactive metabolites: The obtained information on the absorption, distribution, metabolism and excretion of TCC does not indicate a risk for the general population. However, under certain circumstances, i.e. in association with a co-exposure to Ah-receptor agonists and/or a genetically reduced UGT
activity, a higher body burden of the compound alongside with the formation of potentially toxic intermediates could result from TCC exposure.

Our screening for off-target activity of TCC indicates that TCC does not affect the activity of human carboxylesterases, fatty acid amidase, microsomal epoxide hydrolases, cytochrome P450 and glutathione S-transferases. However, we could show with two independent methods that TCC is a potent inhibitor of sEH. With an IC$_{50}$ in the low nanomolar range, TCC exhibits a similar inhibitory potency (IC$_{50}$) as synthetic inhibitors developed as drug candidates.

The main substrates for sEH in vivo are epoxy fatty acids (FA), which are hydrolysed to dihydroxy FA (Morisseau & Hammock, 2013) (Fig. 12). Since epoxy-FA of arachidonic acid and other polyunsaturated FA (PUFA) are potent lipid mediators, sEH plays a key role in the regulation of different biological functions (Morisseau & Hammock, 2013). Stabilization of epoxy FA by sEH inhibition leads to vasodilating, antiinflammatory and analgesic effects in various animal models. Hence, sEH inhibitors are one of the most promising classes of new drugs under investigation for the pharmacological therapy of widespread diseases such as hypertension, atherosclerosis or chronic pain (Duflot et al, 2014; Morisseau & Hammock, 2013). However, as is the case for all pharmacological modulators of the complex arachidonic acid (AA) cascade (Fig. 12), an altered formation of the potent lipid mediators may cause unintended side effects:

![Scheme of the major pathways of the arachidonic acid cascade.](image)

**Fig. 12:** Scheme of the major pathways of the arachidonic acid cascade.
For example, it has recently been shown that the epoxides of AA are pro-angiogenic (Panigrahy et al, 2012). Though this could be a beneficial effect e.g. in cases of wound healing, an increase of AA epoxide levels, e.g. by sEH inhibition, may also promote tumor growth under certain circumstances (Zhang et al, 2014).

In order to investigate if exposure with TCC can alter sEH activity in vivo, we analysed the plasma concentration of epoxy fatty FA (sEH substrate) and dihydroxy FA (sEH product) of TCC-exposed rats by means of LC-MS-based targeted metabolomics of the AA cascade (Fig 12). Rats were treated with a similar topical dose (1 mg/kg body weight) as expected for humans following a whole body shower with TCC containing soap as well as with a high dose of TCC (4 mg/kg body weight). Despite the high potency of TCC as an sEH inhibitor, the treatment did not lead to the systemic inhibition of sEH, as revealed by the oxylipin profiles. Thus, it seems that the systemic concentrations of TCC reached in humans through showering may not be sufficient to cause adverse side effects through sEH inhibition.

Higher (internal) TCC doses (5 mg/kg body weight, intraperitoneally) clearly caused a systemic sEH inhibition, changes in the epoxy/diol FA ratio and downstream biological effects in a murine model of sepsis (Liu et al, 2011). This finding is particularly interesting, since TCC inhibits murine sEH with a 15-fold lower efficacy than the human enzyme (Liu et al, 2011).
Table 3: Rough estimation of the No Observed Effect Level (NOEL) of TCC for systemic sEH inhibition in humans based on a lowest effective dose of 5 mg/kg BW in mice (Liu et al, 2011). Standard extrapolation factors (Nielsen et al, 2008) were used except for the extrapolation of toxicodynamic differences between mice and men where experimentally information was available.

<table>
<thead>
<tr>
<th>dose</th>
<th>Lowest Observed Effect Level (LOEL) 5 mg/KG BW in mice</th>
<th>Estimated No Observed Effect Level (NOEL) 5 mg/KG BW in mice</th>
<th>Estimated No Observed Effect Level (NOEL) 5 mg/KG BW in men</th>
</tr>
</thead>
<tbody>
<tr>
<td>extrapolation factor (LOEL→NOEL) 10</td>
<td>5 mg/kg BW</td>
<td>0.5 mg/kg BW</td>
<td>8 µg mg kg BW</td>
</tr>
<tr>
<td>Standard interspecies extrapolation factor mouse → men (toxicokinetic) 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interspecies extrapolation factor mouse → men (toxicodynamic, sEH inhibition potency) 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extrapolation factor for intra-species differences 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated NOEL</td>
<td>0.8 µg/kg BW</td>
<td></td>
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</tr>
</tbody>
</table>

Based on the pronounced differences in the pharmacodynamics/toxicodynamics of compounds between animals and humans, it is reasonable to expect that the effective dose in humans is significantly lower. This is highlighted by a rough estimation of the Effect Level (NOEL) in Table 3. For the estimation, an extrapolation factor of 10 for intra-species differences was used. This factor might not be sufficient (i.e. the difference might even be greater), since 10-fold inter-individual differences were per se observed when measuring the TCC levels in urine and blood after the same exposure (see above). With this extrapolation factor the roughly expected NOEL is below an internal (absorbed) dose of 1 µg/mg/KG.

Following a 15-min shower with TCC containing soap, the subjects absorbed a dose of about 0.5 mg per person, which corresponds to a dose of about 6 µg/kg body weight. This absorbed dose is in the range of the expected NOEL of TCC in humans.

It has to be taken into account that the calculation of the absorbed dose based on the urinary excretion and even more the calculation of the NOEL based on a LOEL are just rough estimations. However, the fact that both values are within the same
order of magnitude highlights that there is a real possibility of an off-target effect of TCC in humans. Compared to the endocrine disrupting properties of TCC, which were described at high concentrations (Ahn et al, 2008; Chen et al, 2008), sEH inhibition might occur at human exposure-relevant doses. However, the exposure following a standard shower seems not to be sufficient to elicit systemic sEH inhibition. Consistently, administration of comparable topical doses did not change the systemic sEH activity in rats (see above). However, local dermal effects through sEH inhibition cannot be ruled out.

Taking into account that sEHIs potently alleviate inflammatory and non-inflammatory pain (Inceoglu et al, 2011; Inceoglu et al, 2012), one could hypothesize that TCC containing cosmetics might provide pain relief. In this context the combination of sEHIs with non-steroidal antiinflammatory drugs could be a promising strategy for the treatment of (non-inflammatory) diabetic neuropathy as currently tested in a human clinical trial (Human Clinical Trial Number: CTRI/2012/10/003036; http://ctri.nic.in, accessed 23.05.2014).

Treating pain or modulating inflammatory responses (Liu et al, 2011) clearly is one of the most relevant fields of application and mode of action of pharmaceuticals. That these effects are even considered to occur after normal use of TCC containing products indicates that a careful risk-benefit analysis of TCC in personal care products should be undertaken. Particularly, the legal limit of up to 1.5% in cosmetics in the US as well as in the EU should be re-valuated.
The antibacterial soap additive triclocarban (TCC) is widely used in personal care products. TCC shows a high environmental persistence in the aquatic environment and possesses the potential to act as an endocrine disruptor in mammals. In the present work we developed analytical methods for the detection of TCC and its metabolites and applied them to determinate human exposure and bioconcentration in fish. Moreover, metabolism and off-target effects on human enzymes were investigated to assess potential effects of TCC on human health. Using a new sensitive and rapid online solid phase extraction-LC-MS/MS method and a newly developed immunoassay, levels of TCC in urine and blood were quantified following exposure by showering of human subjects with a commercial bar soap containing 0.6% TCC. Starting from a temporary topical dose of 70 ± 15 mg TCC from soap, a low but significant dose of about 6 µg/kg body weight was absorbed by the subjects. TCC blood levels reached a concentration of up to 500 nM. Elimination of TCC required more than 72 hours; however no accumulation was observed after repeated exposure by showering.

The tested Medaka fish bioconcentrate TCC from the surrounding water by about 700-fold, which is lower than expected based on the chemical properties of the highly lipophilic halogenated hydrocarbon. After transferring the fish into fresh water, the compound was rapidly eliminated with a $t_{1/2}$ of 1 h.

In humans and fish TCC was excreted in form of its metabolites, and it is concluded that higher organisms possessing an efficient phase I and phase II metabolism do not accumulate TCC, as earlier described for snails and algae. Oxidative conversion by cytochrom-P450 monooxygenases led to the hydroxylated metabolites 2'-OH, 3'-OH, 6-OH-TCC and 3,4-dichloro-4'-hydroxy-carbanilide (DHC), which are rapidly conjugated by various UDP-glucuronosyltransferases. Renal excretion of TCC almost exclusively occurs in form of its direct N-glucuronides. UGT1A9 was the only UGT isoform that catalyzed this direct conjugation.
The ortho and para hydroxylated phase I metabolites of TCC can be further oxidized to electrophilic reactive quione imines, which covalently bind glutathione (GSH) and proteins. However, the formation of protein adducts was found to be very low in keratinocytes showing significant CYP activity. Upon co-treatment with an Ah-receptor agonist a substantial - but still low - level of protein adducts was detected in the skin cells. The inducible isoform CYP1A1 was found to catalyze the metabolic activation of TCC via dehalogenation and hydroxylation to DHC and oxidation to the para-quione imine. It is concluded that co-exposure of Ah-receptor agonists such as polycyclic aromatic hydrocarbons and TCC may lead to the formation of reactive metabolites in skin, which raises the possibility of skin sensitization.

By using a newly developed LC-MS based high-content screening method it was shown that TCC is an inhibitor of the soluble epoxide hydrolase in the nanomolar concentration range. However, a topical administration of TCC to rats at a dose that is similar to the concentration of TCC to which humans are exposed during showering with TCC containing soap did not alter plasma biomarkers of sEH activity. Nevertheless, there is only a small margin between the absorbed doses in animal studies causing a systemic sEH inhibition and downstream biological effects and exposure levels. Moreover, local dermal effects of TCC containing cosmetics are likely, if one takes into account the high sEH inhibitory potency of the compound together with its high concentration in cosmetics.

The findings indicate that a careful risk-benefit analysis of TCC in personal care products should be undertaken. In particular, long term exposure studies in man, which include biomonitoring of TCC and sEH activity in blood and skin should be carried out to evaluate if the exposure level reached when using PCPs containing up to 1.5% TCC might pose a health risk. With the analytical methods method described herein, we provide excellent analytical tools for these studies.
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LIST OF NON-STANDARD ABBREVIATIONS:

ACN    acetonitrile
BCD    biochemical Detection
BAF    bioaccumulation factor
BSA    bovine serum albumin
BW     body weight
CYP    cytochrome P450 monooxygenase
DHC    3,4-dichloro-4’-hydroxyl carbanilide
DTT    dichlorodiphenyltrichloroethane
EC     electro chemistry
EPA    US Environmental Protection Agency
ESI    electrospray ionization
EU     European Union
FAAH   fatty acid amide hydrolase / microsomal amidase
GSH    glutathione
GST    glutathione S-transferase
GUS    β-glucuronidase
hCe    human esterases
HLM    human liver microsomes
HPRT   hypoxanthine-guanine phosphoribosyltransferase
HRMS   high resolution MS
LC     liquid chromatography
LOD    limit of detection
LOEL   lowest observed effect level
LOQ    limit of quantification
mEH    microsomal epoxide hydrolase
MLM    mouse liver microsomes
MS     mass spectrometry
MS/MS  tandem MS
m/z    mass to charge ratio
NOEL   no observed effect level
PCP    personal care product
PHOME  (3-phenyl-oxiranyl)-acetic acid cyano-(6-methoxy-naphthalen-2-yl)-methyl ester
PPB    parts per billion (µg/kg)
PUFA   polyunsaturated fatty acid
RLM    rat liver microsomes
s/n    signal to noise ratio
SIK    spontaneous immortalized keratinocytes
SPE    solid phase extraction
TCC    3,4,4’-trichlorocarbanilide, triclocarban
TCDD   2,3,7,8-Tetrachlorodibenzo-p-dioxin
UDP    uridine diphosphate
UGT    UDP-glucuronosyltransferase
US     United States of America
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**Manuscript:** Schebb, Inceoglu, Morisseau, Gee, Schebb


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Research design: Schebb, Rice

Experiments and data analysis: Schebb, Buchholz, Rice

Manuscript: Schebb, Hammock, Rice


Research design: Schebb, Buckpitt, Rice

Experiments and data analysis: Schebb, Muvvala, Morin, Buckpitt, Hammock, Rice

Manuscript: Schebb, Buckpitt Hammock, Rice


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Experiments and data analysis: Schebb, Huby, Hwang, Morisseau, Hammock

Manuscript: Schebb, Morisseau, Hammock


Research design: Schebb, Morisseau, Hammock

Experiments and data analysis: Falck, Schebb, Prihatiningtyas, Zhang, Heus, Morisseau, Kool

Manuscript: Falck, Schebb, Prihatiningtyas, Zhang, Heus, Morisseau, Kool, Morisseau, Hammock, Niessen
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