EXTERNAL SCIENTIFIC REPORT

Combined toxicokinetic and *in vivo* genotoxicity study on *Alternaria* toxins[1]

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ABSTRACT

The objective of this project was to gain information on toxicokinetic behaviour and *in vivo* genotoxicity of alternariol (AOH) in NMRI mice. Therefore, commercially available AOH was purified using a preparative HPLC-method. Initial dose-range finding proved AOH to be nontoxic after single or repeated oral application of up to 2000 mg/kg (limit dose). Subsequently, an *in vivo* oral toxicokinetic study (OECD guideline 417) was performed with 200 and 1000 mg/kg radiolabelled AOH. The study revealed low systemic absorption, with about 90 % of the total dose excreted via faeces and up to 9 % via urine. Blood levels did not exceed 0.06 % of the administered dose during the first 24 h after administration. Thus, target organ toxicity would most likely be restricted to the gastrointestinal tract. Metabolism of AOH was then investigated in a toxicokinetic study with non-radiolabelled AOH. Three dosage schemes were used: 200 and 2000 mg/kg (single dose) and 3 x 2000 mg/kg (0, 24 and 45 h). Whole blood (LC-MS/MS analysis) and urine (GC-MS in SIM mode) were analysed for AOH and its hydroxy-metabolites. Four metabolites (8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH, 2-hydroxy-AOH) were detected and ID was confirmed by NMR and mass spectrometry. Results also pointed to low systemic absorption, but mean blood levels (0.5 µM AOH, 3 h after the last of three applications) was considered sufficient to justify performance of a combined bone marrow micronucleus test (OECD 474) and *in vivo* alkaline comet assay (stomach, gut, liver). Therefore, 3 x 2000 mg/kg AOH were applied in corn oil (at 0, 24, and 45 h) and animals were sacrificed 48 h after the first application. The micronucleus assay revealed no toxic or genotoxic effect of AOH in bone marrow and the comet assay with liver tissue also did not indicate systemic genotoxicicity.

KEY WORDS

alternariol, toxicokinetics, genotoxicity, metabolism

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SUMMARY

During the course of the study it became clear that sufficient amounts of AOH would not be commercially available to conduct the study in rats as initially planned. Thus, it was agreed with EFSA to perform the study in NMRI mice, which required less AOH.

AOH was chemically synthesized by J. Podlech (Karslruhe) and subsequently purified by liquid chromatography at Fraunhofer ITEM resulting in AOH material with a ^{1}H NMR-purity of 93.4 % (>98 % HPLC). According to the ^{1}H NMR quantification the 3.4 g lot contained 93.4 ± 0.7 % AOH and 1.3 ± 0.06 % ethyl acetate. All the rest consists mostly of water, which corresponds to an HPLC purity of > 98 %.

A combined dose-range finding for the radiolabelled toxicokinetic study and the micronucleus test *in vivo* was done as first step. Male and female NMRI mice (1m/1f per dose), which were approximately 8 -9 weeks old, were treated orally with increasing doses of AOH (50, 100, 300, 1000 and 2000 mg/kg body weight (BW)). Over an observation period of one week the study subjects were monitored daily for clinical signs and their body weights were recorded. No effect of the administration of AOH on the general health status was observed during the 7-day observation period, at any dose. The limit dose of 2000 mg/kg was then repeatedly applied at 0, 24 and 45 h to one male and one female mouse. Again, no treatment-related effects were observed.

Those initial experiments were followed by a toxicokinetic study according to OECD guideline 417 using radioactive AOH. Synthesis of [carbonyl-^{14}C] AOH was done at Quotient Bioresearch (Cardiff, UK). Briefly, a total of 16 male NMRI mice were treated with 1000 mg/kg BW (high dose) or 200 mg/kg BW (low dose) at 1.6 MBq/animal radiolabelled 6-^{14}C-AOH by oral gavage (single dose). Radioactivity was then periodically measured in excreta over a time period of 168 h using 24 h sampling intervals. After sacrifice of the study subjects (after 24 h and after 7 days) selected tissues were analysed for radioactivity. The fairly high radioactivity values detected in faeces revealed that the systemic absorption of AOH in mice after oral gavage is rather low. Faecal excretion accounted for 84.5 ± 6.4 % in the high dose group whereas 90.7 ± 4.3 % were recovered in the faeces in the low dose group. In the urine excreted over a time period of one week after administration, a total of 5 to 11 % of the administered dose was detected. The mean values were 9.3 ± 3 % and 5.7 ± 0.7 % in the high and the low dose group, respectively. Blood radioactivity levels were measured at 2, 6 and 24 h after administration. During this sampling period the total radioactivity recovered in blood did not exceed 0.06 % of the dose applied. Only trace amounts were detected in exhaled air, the levels being 0.05 ± 0.01 % and 0.03 ± 0.01 % in the high and the low dose group, respectively. Tissue distribution studies confirmed that the absorption of AOH after oral gavage was low. Tissues analysed for radioactivity included heart, lung, liver, spleen, stomach, small intestine, large intestine, muscle, kidneys, testes, renal fat, brain and bone. As expected the highest radioactivity levels were detected in the gastrointestinal tract 24 h after oral application. However, in all organs and tissues (including the gastrointestinal (GI) tract) investigated 24 h after application, the radioactivity levels accounted for less than 1 % of the applied dose and this fraction declined to less than 0.01 % at day 7. It was therefore concluded that the main proportion of orally administered AOH is most likely excreted via faeces without being absorbed. Hence, whole blood and urine were selected as compartments of choice to investigate the toxicokinetics and metabolism of AOH in the scope of a non-radioactive study in NMRI mice. A non-radioactive toxicokinetic study was done on 18 male and 18 female NMRI mice to address the kinetics and metabolism of AOH. Seventeen male and 17 female mice were dosed with 200 mg/kg BW and blood samples were taken after 0.5, 1, 2, 3, 4, 5, 6, 24 and 48 h for the determination of AOH blood levels. Five male and 5 female mice were housed in metabolism cages after applications, where urine was collected for 72 h and subsequently analysed for AOH and its potential metabolites.

In addition, the whole blood of five animals from the combined genotoxicity study was analysed for AOH metabolites. These animals received three times the limit dose of 2000 mg/kg at 0, 24 and 45 h and were sacrificed 48 h after the first application. Due to the high lipophilicity of AOH, intravenous application was impossible. Unlike initially planned, to enable the calculation of oral bioavailability, an intravenous treatment group could not be included in the study.

For the quantification of AOH in mice whole blood a LC-MS/MS analytical method using matrix matched standards and 7-Hydroxy-4-phenylcoumarine (HPC) as internal standard was developed. Furthermore, a GC-MS analytical method using matrix matched standards and 6,7-Dihydroxycoumarin (DHC) as internal standard was developed and applied for the quantification of AOH in mice urine. The oxidative metabolism of AOH was mimicked by rat liver microsomal incubations. In this incubation batch four oxidative metabolites of AOH, namely 8-hydroxy-AOH (O1), 4-hydroxy-AOH (O2), 10-hydroxy-AOH (O3) and 2-hydroxy-AOH (O4) were detected and their ID confirmed by ^{1}H NMR spectroscopy and mass spectrometry. This *in vivo* incubation proved useful as it assisted the unambiguously determination of those four metabolites in mice urine. The total urinary excretion of AOH and these four metabolites during the 72 h collection period accounted for 0.1 to 0.6 % of the administered dose (200 mg/kg BW). This is in contrast with the results obtained with radiolabelled AOH, which suggested that 9 % of the applied (radioactive) dose were excreted via urine. A plausible explanation for this discrepancy could be that other metabolites or breakdown products contributed to the radioactivity recovered in urine.

In the non-radioactive study the AOH blood levels were in the two-digit ng/mL range during the first 3 to 6 hours after AOH administration of 200 mg/kg BW. C_{max} levels were comparable between males and females accounting for 66.2 and 90.2 ng/ml respectively. T_{max} was reached after 2 h in males and after 0.5 h in females. The time course of AOH blood concentrations suggested a slightly faster clearance in males with a half-life (t½) of 1 h while the blood half-life was 9 h in females. Three AOH metabolites were found in blood, namely 4-hydroxy-AOH, 10-hydroxy-AOH and 2-hydroxy-AOH. Since the study had to be performed in mice, only small volumes of blood and urine were available for analysis. The search for phase II metabolites was thus beyond the scope of this study. However, we hypothesize that phase II metabolites, e.g. glucuronide and/or sulphate conjugates, are formed and excreted via urine. This would also explain the different results observed for urinary excretion in the radioactive and the non-radioactive study.

The *in vivo* alkaline comet assay had to be adapted to mice. Single cell isolation from liver, stomach, and gut of male NMRI mice as well as an appropriate positive control with respective dose and treatment schedule had to be established. Therefore, a pre-study was performed with six negative and six positive control animals. In the negative control animals the highest mean (of the medians, 2 slides per animal, 50 cells per slide) tail intensities (TI) were observed for gut (8.7 ± 2.19 %), followed by stomach (2.0 ± 0.87 %), blood (0.1 ± 0.03 %), and liver (0.1 ± 0.02 %). EMS was demonstrated to significantly induce mean TI in blood, liver, stomach, and gut, when applied in a dose of 300 mg/kg 4 h before sacrifice. The highest effects were observed in gut (mean TI: 21.5 ± 4.39 %), followed by stomach (mean TI: 6.1 ± 0.29 %), blood (mean TI: 3.8 ± 0.78 %), and liver (mean TI: 3.0 ± 0.45 %), indicating highest effects in the tissues of first contact after oral application. After this pre-study, a respective GLP-compliant standard operating procedure (SOP) was issued including scraping, followed by mincing of the tissues for single cell isolation and considering the EFSA “Minimum criteria for the acceptance of *in vivo* alkaline comet assay reports”.

To investigate the possible genotoxic potential of AOH *in vivo*, a mammalian erythrocyte micronucleus test was performed (according to OECD guideline No. 474) with bone marrow of male NMRI mice, in combination with an *in vivo* alkaline comet assay with significantly exposed tissues

(stomach, gut) and a systemic organ (liver). A limit test design was chosen, using an application scheme of 3 x 2000 mg/kg AOH (in corn oil) at time points 0, 24, and 45 h with sacrifice of the animals 48 h after the first application. This application scheme resulted in mean blood concentrations of about 0.5 µM AOH 3 h after the last application, as determined for five animals in the scope of the toxicokinetic study. The micronucleus test was done in compliance with the Principles of Good Laboratory Practice (GLP). The test item AOH thereby did not induce treatment-related mortality or significant signs of acute toxicity in male NMRI mice. There was, however, slight reduction in mean body weight between the first and last day of application for both corn oil and AOH-treated animals in the micronucleus test, amounting to -0.54 ± 1.232 g and -1.04 ± 1.100 g, respectively. These reductions in body weight were not statistically significant. In contrast to those finding, cyclophosphamide monohydrate (CP)-treated animals demonstrated body weight gain. As assessed by differential cell counts of bone marrow smears, AOH in the given dose did not significantly influence red blood cell (RBC) formation in male NMRI mice. The mean ratio of polychromatic erythrocyte (PCE) to the whole RBC fraction amounted to 111 ± 9.8 PCE/200 RBC, compared to 107 ± 14.2 PCE/200 RBC for the vehicle control. The positive control CP also demonstrated no repression of RBC formation, with a mean number of 107 ± 9.6 PCE/200 RBC. Under the specific treatment conditions used, AOH did not significantly enhance the number of micronuclei in PCE of the bone marrow of male NMRI mice and is thus considered non-mutagenic in immature bone marrow erythrocytes (PCE) of male NMRI mice. In line with these data, the *in vivo* alkaline comet assay with liver tissue did not indicate a systemic genotoxic potential of AOH. Group mean TI (of the medians, 3 slides per animal, 50 cells per slide; n = 10) of AOH-treated animals (0.93 ± 1.417 %) resembled that of the corn oil-treated vehicle controls (0.94 ± 0.480 %; n = 10) with, however, a slightly higher amount of hedgehogs in the AOH group. The *in vivo* alkaline comet assay with stomach, and in particular with gut tissue, which was performed to investigate local genotoxicity in the gastrointestinal tract, could not adequately be analysed, due to an obviously adverse effect of repeated corn oil exposure of the animals on tissue and slide preparation for the comet assay. Unexpectedly, high mean TI, in particular in the corn oil-treated vehicle controls, was observed. For stomach tissue mean TI for corn oil-treated animals was >> AOH > EMS. Concerning gut, the vehicle control slides were mostly not analysable (< 5 animals). The slides of AOH-treated animals were somehow better, but also exhibited unexpectedly high mean TI, whereas the EMS (applied in water)-treated positive controls were comparable to the pre-study, with a mean tail intensity of 21.08 ± 10.526 %. Thus, in the absence of significant treatment-related histopathologic changes, corn oil treatment, possibly in combination with overnight starving, seemed to interfere with tissue/slide preparation from stomach and gut. This might be due to a decreasing resistance of cells to mechanical and/or chemical stress. Consequently, investigation of local genotoxicity of AOH in the GI by using the comet assay seems only be possible by using other application modes than oral gavage in corn oil.

In conclusion, the genotoxic potential of AOH observed *in vitro* could not be confirmed in the mammalian erythrocyte micronucleus test *in vivo* with bone marrow of male NMRI mice.

The lack of AOH toxicity is in line with recent studies, suggesting that AOH plays only a minor role for the genotoxic potential of *Alternaria* extracts (Fleck et al., 2012; Schwarz et al., 2012). It is thus recommended to study the mechanism of action of other potentially as yet unidentified *Alternaria* mycotoxins (e.g. altertoxin II) in more detail.

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TERMS OF REFERENCE AS PROVIDED BY EFSA

BACKGROUND

In 2011, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) adopted a Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food.

Alternaria toxins are mycotoxins produced by *Alternaria* species, fungi widely distributed in the soil microflora, which can act both as saprophytes and plant pathogens. *Alternaria* species are principal contaminating fungi in wheat, sorghum and barley, and have also been reported to occur in oilseeds such as sunflower and rapeseed, and in several fruits and vegetables. Namely, *Alternaria alternata* is the most common *Alternaria* species found in fruits and vegetables and is the most important mycotoxin-producing species.

Alternaria species produce more than 70 secondary metabolites, but only few of them have been chemically characterised and are known to cause toxic effects both in humans and animals.

In particular, some *Alternaria* toxins and culture extracts of *A. alternata* have shown positive genotoxic results when tested in mutagenicity and clastogenicity *in vitro* assays both in bacteria and mammalian cell systems. Among these, alternariol (AOH, CAS No. 641-38-3) and alternariol monomethyl ester (AME, CAS No. 23452-05-3) were reported to cause mutagenicity both in bacterial systems (Kada et al., 1984; An et al., 1989; Zhen et al., 1991; Schrader et al., 2006) and in mammalian systems (Dong et al., 1993; Brugger et al., 2006) and clastogenic damages in a series of mammalian cell cultures (Liu et al., 1992; Lehmann et al., 2006; Pfeiffer et al., 2007a). Altertoxin-I (ATX-I, CAS No. 56258-32-3), -II (ATX-II, CAS No. 56257-59-1) and –III (ATX-III, CAS No. 105579-74-6) showed gene mutations when tested in an Ames assay (Stack and Prival, 1986) and ATX-I and ATX-III induced cell transformation in murine fibroblasts and in an immortalised human cell line (Osborne et al., 1988). Finally, a recent study indicated a plausible genotoxic mode of action for AOH, showing that it can act as an inhibitor of DNA topoisomerase I and II (Fehr et al., 2009).

Limited information shows that AOH can be extensively absorbed by the gastrointestinal system (Burkhardt et al., 2009), whereas a poor absorption was observed both *in vitro* and *in vivo* for AME (Burkhardt et al., 2009; Pollock et al., 1982). Following absorption, rapid metabolism was observed for AME (Pollock, 1982). Metabolite characterisation was undertaken both for AOH and AME. Both toxins showed oxidative metabolism pathways leading to formation of catechols or hydroquinones, and possible glucuronide- and sulphate-conjugates (Pfeiffer et al., 2007b, 2009; Burkhardt et al., 2009).

In the scientific opinion, the CONTAM Panel recommended the generation of additional genotoxicity data for most of the *Alternaria* toxins and in particular the generation of toxicity data for AOH and AME enabling their risk assessment. Moreover, the CONTAM Panel noted that no reliable information is available on the toxicokinetics and metabolism of the toxicologically relevant toxins and recommended that data should be generated also in this respect.

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OBJECTIVES

The present call aims to generate data to fill the information gaps in the fields of toxicokinetics and genotoxicity and allowing for the refinement of the risk assessment on *Alternaria* toxins. The Scientific Opinion indicated that, among the potentially genotoxic *Alternaria* toxins, AOH occurs with a relatively high frequency in foodstuffs. In particular, AOH has shown positive results when tested in mutagenicity and clastogenicity assays *in vitro* both in bacteria and mammalian cell systems. No *in vivo* genotoxicity data were retrieved to confirm the genotoxic potential of those *Alternaria* toxins.

Moreover, preliminary *in vitro* data shows a high intestinal absorption potential for AOH (Burkhardt et al., 2009). In conclusion, AOH is selected as the representative *Alternaria* toxin to be tested.

The objective of the project is to conduct a combined study for the determination of the toxicokinetic profile and the assessment of genotoxicity of AOH in rodents.

TASKS

An *in vivo* toxicokinetic (TK) study in rodents shall be performed to characterise the kinetic profile of AOH. The toxicokinetic study shall include the synthesis of radioactivity labelled-AOH and shall be aimed at the characterisation of the following aspects:

- The determination of the rate and extent of gastrointestinal absorption, including the estimation of the following parameters: systemic bioavailability (f), peak concentration in plasma/blood (C_{max}), time of peak concentration in plasma/blood (t_{max}), following oral exposure;

- The distribution of AOH in the body, including the determination of the following kinetic parameters: volume of distribution (Vd) and blood/plasma elimination half life ($t_{1/2}$). Distribution will be determined at least in the following tissues/organs: liver, kidney, spleen, gastrointestinal tract, reproductive organs and brain;

- The metabolism of AOH, including the identification and chemical characterisation of the main metabolites in blood, urine and faeces by means of adequate analytical techniques. The metabolic pathway scheme should be also provided. Supporting *in vitro* experiments can also be included, if adequate.

- The excretion of AOH, including the recovery rate of the parent compound and the main metabolites from urine and faeces up to 96 hours after the end of the treatment.

An *in vivo* genotoxicity study in rodents. The battery of genotoxicity assays to be included in the *in vivo* study shall be selected on the grounds of the available *in vitro* data indicating a genotoxic potential for AOH and shall be justified by a clear testing strategy rationale. In particular:

- The selected battery of genotoxicity assays shall cover both the mutagenicity and clastogenicity endpoints.

- The assays shall be performed to enable the evaluation of genotoxicity at the site of contact of the substance following ingestion (e.g. oesophagus mucosa) and at the critical organs/tissues following systemic absorption. The information gathered from the TK study could be used to support the genotoxicity testing strategy.

Both studies shall be conducted in compliance with commonly accepted toxicological testing guidance documents and with the principles of good laboratory practices (GLP).

The results of the studies will be described in a Final Technical Report. Interim and Final Technical Reports will be prepared in line with the time schedule reported in I.1.4 of the technical specifications (ToR).

This contract was awarded by EFSA to:

Contractor: Fraunhofer Gesellschaft zur Förderung der angewandten Forschung e.V.

Contract title: Combined toxicokinetic and *in vivo* genotoxicity study on *Alternaria* toxins

Contract number: OC/EFSA/CONTAM/2012/01

INTRODUCTION

Only limited information is available on the bioavailability and toxicokinetics of AOH after oral application. Furthermore, *in vivo* genotoxicity data confirming the genotoxic potential observed *in vitro* are lacking. It was thus the objective of this study to gain information on the toxicokinetic behaviour and on the *in vivo* genotoxic potential of AOH in mice.

MATERIALS AND METHODS

1. Up scaled oxidative *in vitro* metabolism of AOH (microsome incubation)

Oxidative *in vitro* metabolites of AOH were generated by incubating 3.1 mL microsomes (20 mg/mL Rat Microsomes Pool GIBCO 50 % Glycerin No.: RTMCPL) dissolved in 3.1 mL 0.1 M phosphate buffer with 50µM Alternariol and a nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH)-generating system (179µL (433U/mL) isocitrate dehydrogenase, 698 µL 2.33M isocitrate, 195 mg NADP+, and 775 µL 1M magnesium chloride in 4264 µL phosphate buffer) in a final volume of 142 mL of 0.1 M phosphate buffer at pH 7.4. After pre-incubation for 30 min at 37 °C, the 6.2 mL NADPH-generating system and 6.2 mL of the microsomes were added and the mixture incubated for 5 min at 37 °C. Then 0.77 mL 26.9 mM Alternariol was added and the mixture incubated for additional 90 min at 37 °C. Subsequently, the incubation mixture was extracted with 1:1 ethyl acetate and the pooled extract evaporated to dryness. The residue was dissolved in suited solvent either for MS or NMR analyses.

2. Purification of synthesised Alternariol by preparative chromatography

2.1. Preparative liquid chromatography

Chromatographic purification was carried out as previously described.

The raw AOH dissolved in the eluent was purified isocratically on a C18 column.

Eluent (isocratic):	THF 26 %/MeOH 30 %/H_2O 44 %, 0.01 % TFA
Flow:	20 mL/min
Concentration:	Stock Solution: 0.5 g/100 mL
Appl. Volume:	10 mL (ca. 50 mg Alternariol/10 mL eluent)
Detection:	UV; 254 nm
Range:	0 – 0.250 Abs (= 10 mV)
Collected fraction:	Fraction 1-1: from minute 7 to signal decrease to 0.100 Abs (= 4 mV) after about 14 min

The collected LC-fractions were extracted with 1:1 ethyl acetate and the pooled extract evaporated in a rotary evaporator to dryness. It was noted that too long drying led to brownish discoloration (oxidation of the phenolic hydroxyl groups) of the product.

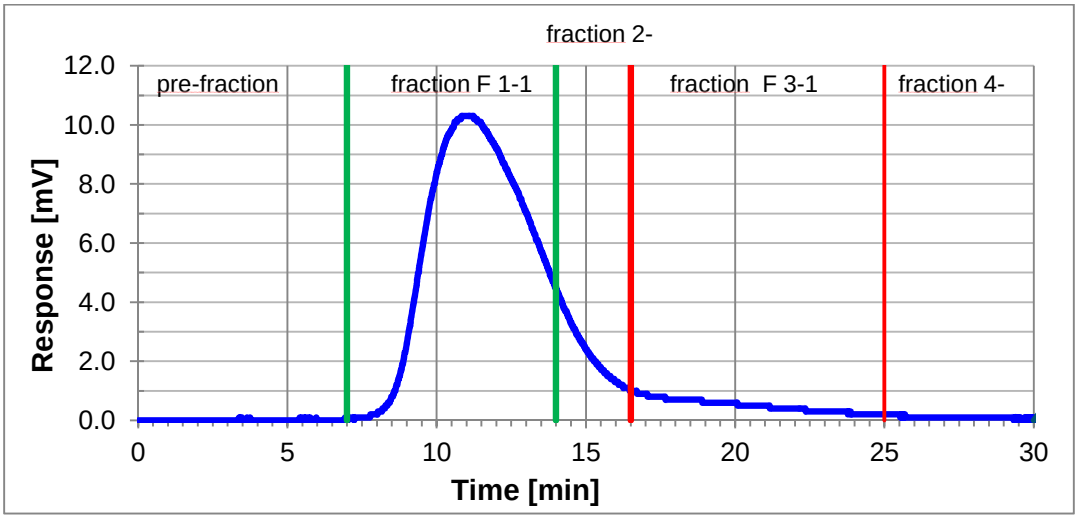


Figure 1 UV-Chromatogram of the preparative purification of synthesized AOH

2.2. Analytical liquid chromatography

The HPLC separations were performed using Agilent-1100 HPLC with degasser, quartery pump, auto-sampler, and Bruker-DAD detector (200-400 nm, trace 251-259 nm, l = 2 mm).

Eluent A: 0.012 % formic acid, pH= 3.5

Eluent B: pure acetonitrile

Gradient: start 18 % B, 10 min 42 % B, 17.5 min 48 % B, 24 min 90 % B.

Flow: 0.7 mL/min

Column: Agilent Eclipse 150x4.6 mm, 5 µm particle size

Injection volume: 5-30 µL

2.3. Microsomal incubations with alternariol

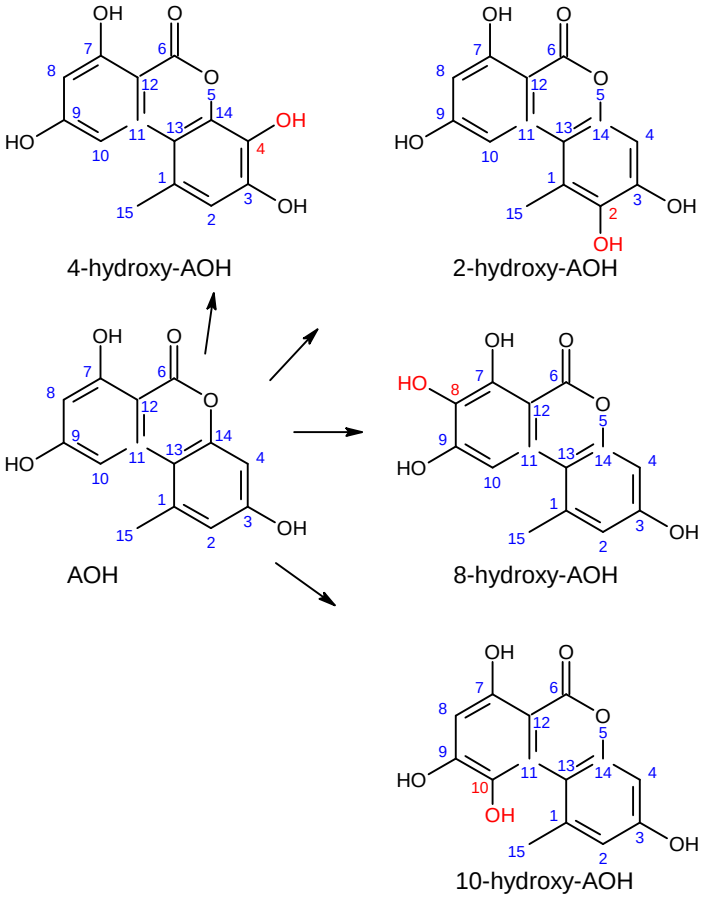


Figure 2 Metabolism scheme

Microsomal incubations with alternariol and subsequent extraction of AOH and its hydroxylated metabolites were carried out as follows:

Materials and Chemicals

Microsome source:	rat liver
Dipotassium phosphate buffer (0.1 M)	
Magnesium chloride solution (1.0 M)	
Isocitrate buffer (2.33 M)	
Isocitrate Dehydrogenase (200 U / mL):	Isocitrate dehydrogenase 150 U = 1 pckg unit (200 U / mL = 200 U / 1000 µL = 150 U / 750 µL)
NADP+:	weighed portion: 194 mg
Alternariol stock solution:	3.8 mg/mL in DMSO

Microsomal Incubation	
Total volume:	approx. 153.7 mL
Protein concentration:	approx. 1 mg/mL
Incubation temperature:	37 °C

Incubations:

1. Dipotassium phosphate buffer (0.1 M; 141.09 mL) is transferred into an autoclaved glass bottle and kept in a water bath (shaking, 37 °C) until the buffer has reached a temperature of 37 °C.

2. Protein cocktail: The protein cocktail is prepared freshly on the day by mixing isocitrate buffer (2.33 M; 698 µL), isocitrate dehydrogenase (200 U/mL; 388 µL); magnesium chloride solution (1 M; 775 µL); dipotassiumhydrogen phosphate buffer (0.1 M; 4264 µL) and NADP+ (194 mg).

3. Microsomes (310 µL) are diluted with dipotassiumhydrogen buffer (0.1 M; 5.89 mL) and added together with the protein cocktail to the pre-heated dipotassium phosphate buffer (0.1 M; 141.09 mL; 37 °C). The resulting mixture is incubated in a water bath (shaking; 37 °C) for 5 min.

4. After addition of alternariol stock solution (12.5 mM; 1.55 mL) the mixture is incubated in a water bath (shaking; 37 °C) for 60 min.

5. After the incubation cycle the sample is stored at – 20 °C prior to extraction.

Extraction Procedure:

1. The frozen sample (see point 5 under Microsomal Incubation) is thawed and homogenised using a vortex mixer.

2. DHC-IS working solution A (40 µL; chapter 3.3.4) is added to a sample aliquot (100 µL) in a 2-mL flat bottom glass vial (white, clear).

3. Liquid-liquid extraction is performed by adding ethyl acetate (1 mL) and subsequent mixing for a.) 1 min at 1800 rpm using a vortex mixer or b.) 6 min at 1000 rpm using a thermo mixer (Eppendorf). Separation of the two phases is achieved by letting the sample sit on the work bench for 1-2 min.

4. The top layer is transferred into a clean 2-mL flat bottom glass vial (white, clear). The solvent is removed in a nitrogen stream (approx. 20 min).

5. The resulting dry residue is taken up in methanol (750 µL) and mixed for 30 s at 700 rpm.

6. The extract is stored in a sealed glass vial at approx. 4 °C prior to analysis.

2.4. HPLC peak cut preparation of oxidized metabolites of AOH

The first enrichment step was carried out by repeated extraction with ethyl acetate. The HPLC separations were performed using Hewlett Packard -1100 HPLC with degasser, quartery pump, auto-sampler, and Bruker-DAD detector (200-400 nm, trace 251-259 nm, l = 2 mm). The selected chromatographic peaks of the oxidative metabolites were collected repeatedly in separate collecting vessels. All HPLC fractions of interest were collected, extracted with ethyl acetate, and used for MS and NMR analyses.

3. AOH identity and purity confirmation by NMR spectroscopy/mass spectrometry

NMR spectroscopy: All samples were characterized by NMR spectroscopy using a high-performance digital 600-MHz NMR spectrometer Avance III (by Bruker) equipped with a 5-mm TCI cryoprobe head ($^{1}H/^{13}C$ inversely, Z-gradient). All NMR spectra were recorded in $CDCl_3$ / DMSO-D_6 (50:50) by using standard pulse sequences. All obtained spectra were referenced to the solvent signal DMSO-D_5 (^{1}H, δ = 2.514 ppm[2], ^{13}C, δ = 39.5 ppm).
^{1}H-NMR-1D spectra: pulse sequence zg30, TD 32K, ns 32, AQ 1.95sec, D1 30sec, sweep width 8420Hz; ^{13}C-NMR-1D spectrum (AOH): pulse sequence zgig30, TD 32K, ns 5120, AQ 1.95sec, D1 3sec, sweep width 33333Hz; $^{1}H/^{1}H$ -NMR-COSY[3]-2D spectra: pulse sequence cosygpqf, TD 2K, ns 2-4, AQ 0.14sec, D1 1.5sec, sweep width 7400Hz; $^{1}H/^{1}H$-NMR-ROESY[4]-2D spectra: pulse sequence roesyph, TD 2K, ns 2-4, AQ 0.14sec, D1 2.0sec, sweep width 7400Hz; $^{1}H/^{13}C$-NMR-HSQC[5]-2D spectra: pulse sequence hsqcetgpsi2, TD 2K, ns 4, AQ 0.14sec, D1 1.5sec, sweep width 7400Hz; $^{1}H/^{13}C$-NMR-HMBC[6]-2D spectra: pulse sequence hmbcgpplpmdqf, TD 2K, ns 16-32, AQ 0.14sec, D1 2.0sec, sweep width 7400Hz.
NMR sample preparation: Dried HPLC cuts were dissolved in 65 µL 50/50 v/v $CDCl_3$/DMSO-D_6 and the solution was transferred into an NMR glass capillary (OD = 2 mm) and fused by melting.

Mass spectrometry: The mass spectrometric (MS) measurements were performed with:

A) a Bruker Esquire LC ion trap mass spectrometer for flow injection. The following setting was used: Nebulizer nitrogen 11 psi, 5L/min; dry temperature 300 °C, mass range 50-600 amu; ICC 30000 max 50 ms; flow 400 µL/h.

B) a Bruker HCT ion trap mass spectrometer for LC-UV-MS measurements. The following setting was used: ESI-MS negative mode, target Mass 250 amu, stability 50 %, scan 50-570 amu, ICC 100000, dry temp 300 °C, nebulizer nitrogen, 15 psi, 8 L/min.

C) an ABSciex 5500 QTrap mass spectrometer for quantitative LC-MS measurements of AOH.

3.1. Determination of the AOH purity (NMR)

6.61 mg AOH and 4.86 mg maleic acid were dissolved in 200 µL 50/50 $CDCL_3$ / DMSO-d6. The ^{1}H NMR spectra were recorded using the Bruker pulse program zg30. The following AOH signals (Px) and the corresponding integrals (Ix) were used for quantification: A proton from C-2, C-4, C-8 and C-10 as well as the three protons of the C-15.

Calculation:
$$\frac{I_x \cdot M_x \cdot m_s \cdot pu_s}{I_s \cdot M_s \cdot 100 \cdot P_x} \cdot 2 = m_x$$

x = sample, s = standard (2 protons), M = molecular mass, I = integral, pu = purity, P= protons, m = mass

3.2. Structural elucidation of the oxidized metabolites of AOH (NMR/MS)

The structural elucidation of the oxidative metabolites was performed with the 1D/2D-NMR and MS methods listed above.

[2] The ^{1}H chemical shift belongs to always present incomplete deuterated DMSO-D_6 (= DMSO-D_5).
[3] COSY = **co**rrelated **s**pectroscop**y**.
[4] ROESY = **r**otating frame **O**verhauser **e**ffect **s**pectroscop**y**.
[5] HSQC = **h**eteronuclear **s**ingle **q**uantum **c**oherence.
[6] HMBC = **h**eteronuclear **m**ultiple **b**ond **c**orrelation.

4. Sample preparation

4.1. Mice whole blood (LC-MS)

K3EDTA mice whole blood (25 µL) was transferred into a 0.5-mL Eppendorf tube. IS intermediate solution B (6 µL) and methanol (5 µL) were added and the sample mixed on a vortex mixer. Proteins were precipitated by adding methanolic zinc sulphate solution (250 µL). The solution was mixed on a vortex mixer for 30 sec and then centrifuged at 10000 rcf for 10 min at room temperature. Methanol (100 µL) was added, the sample mixed on a vortex mixer and then centrifuged at 10000 rcf for 5 min at room temperature. An aliquot (100 µL) of the supernatant was transferred into a 2-mL HPLC-vial, aqueous methanol solution (400 µL, 50 % methanol (aq)) was added and the sample mixed on a vortex mixer prior to LC-MS/MS analysis.

4.2. Mice urine (GC-MS)

A solution of methanol / water 70:30 containing 100 µg internal standard and mice urine (200 µL) were transferred into a 2-mL Eppendorf tube. Ethylacetate (300 µL) was added and the sample mixed on a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was transferred into a clear 2-mL autosampler vial. The residue was extracted with ethylacetate (300 µL) by thorough shaking using a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was combined with the first supernatant and the resulting sample was dried in a nitrogen stream. After complete removal of all solvent and water, BSTFA (40 µL) was added. The resulting mixture was left over night at room temperature, to allow complete derivatisation, prior to GC-MS analysis.

5. Analytical method development and validation (LC-MS / GC-MS)

The method validation was performed in accordance with the guideline of bioanalytical method validation (EMEA/CHMP/EWP/192217/2009). This guideline provides recommendations for the validation of bioanalytical methods applied to measure drug concentrations in biological matrices obtained in animal toxicokinetic studies and all phases of clinical trials. For cost reasons, a guideline-compliant full validation has been omitted. The full validation normally comprises:

- Selectivity
- Carry-over
- Lower limit of quantification
- Calibration curve
- Accuracy
- Precision
- Dilution integrity
- Matrix effect
- Stability

All these crucial points were of course addressed but they were not validated to full extent. In particular, dilution integrity, matrix effect and stability were validated with reduced sample size. The validation was performed only for AOH, since for the oxidative metabolites no standards are available. Although the validation is designed as non-GLP, the principles of GLP as well as the EMA-“Guideline on bioanalytical method validation” [1] were taken into consideration and the validation was conducted in the spirit of GLP. The method validation is given in a supplementary report to this report Appendix H.

5.1. LC-MS analytical method

The quantification of AOH in mice whole blood was achieved by LC-MS/MS analysis in MRM mode using matrix matched standards and 7-Hydroxy-4-phenylcoumarine (HPC) as internal standard. The LC-MS method was carried out with an Agilent-1100 HPLC with degasser, quartery pump, auto-sampler, and Bruker-DAD detector (200-400 nm, trace 251-259 nm, l = 2 mm).

Table 1 HPLC parameters and settings used for AOH determination in mice whole blood

Parameter	**Settings**
System	Agilent 1260
Trap column	Zorbax Eclipse Plus C8, 2.1x12.5 mm, 5 µm ; SN : USUQB03701
Analytical column	Poroshell 120 EC-C18, 3x50 mm, 2.7 µm; SN: USCFZ12555
Column temperature [°C]	50
Injection volume [µL]	10
Autosampler temperature [°C]	7 or uncontrolled
Needle wash	0.1 % formic acid (aq):acetonitrile:methanol:isopropanol (1:1:1:1)
Mobile phase	
Trap column	Isocratic: methanol/water 25:75
Analytical column	**A:** water
	B: methanol

Gradients

Trap column

Time [min]	Flow rate [µL / min]	Valve position	time [min]
0.00	500	right	0.00
0.01	500	left	0.01
1.51	500	left	1.52
1.52	500	right	1.53
22.00	500	right	18.00
		left	18.01
		left	22.10
		right	22.20

Analytical column

Time [min]	Flow rate [µL / min]	B [%]
0.00	500	25
1.00	500	25
10.0	500	80
15.00	500	80
15.01	500	95
18.00	500	95
18.01	500	25
22.00	500	25

Table 2 MS parameters and settings used for AOH determination in mice whole blood

Parameters	**Settings**
System	ABSCIEX QTRAP 5500
Source	*Turbo* V™
Mode	Negative
Capillary voltage [V]	-4500
Curtain gas (N_2) [psi]	30
Gas 1 (N_2) [psi]	50
Gas 2 (N_2) [psi]	70
Temperature [°C]	550
CAD gas	Medium
Dwell time [ms]	150
Entrance potential [V]	-10

MRM transitions

Compound	Q1 m/z	Q3 m/z	Collision energy	CXP	DP
AOH - Quantifier	257	213	-36V	-15V	-60V
AOH – Qualifier	257	147	-46V	-5V	-60V
HPC – Quantifier	237	193	-35V	-15V	-50V

5.2. GC-MS analytical method

The quantification of AOH in mice urine was achieved by GC-MS analysis in SIM mode using matrix matched standards and 6,7-Dihydroxycoumarin (DHC) as internal standard. AOH and its metabolites (Fig. 1) were determined by GC-MS analysis after their derivatisation with N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA). Quantitation was achieved by Single Ion Monitorinig (SIM). The analyte AOH was monitored on the mass to charge ratios (m/z) 459 (quantifier) and 222 (qualifier) and the internal standard DHC on the m/z trace 322. All four metabolites (O1, O2, O3 and O4) were monitored on m/z 547.

Table 3 GC-MS parameters and settings used for AOH determination in mice urine

Parameter	**Settings**
System	HP 6890 (GC) / HP 5973 (MS); Gerstel MPS 2 Autosampler
Mode	Electron impact (EI)
Ionisation energy [eV]	70
Column	HP5ms, length: 30m; diameter: 0.25 mm; film thickness: 0.25 µm
Carrier gas	Helium
Carrier gas flow [mL / min]	1.2 (constant flow)
Injection volume [µL]	1
Injector temperature [°C]	250
Injector mode	splitless
Temperature program	Start: 60 °C for 1 min; End: 290 °C for 15 min; Rate: 15 °C / min
Quadrupole temperature [°C]	150
Data acquisition	
Compound ID	Target (m/z)
AOH	459 (qualifier: 222)
DHC (IS)	322
O1	547
O2	547
O3	547
O4	547

6. Dose range finding study

Male and female NMRI mice, approximately 7-8 weeks of age, were purchased from Charles River. Mice were treated orally with increasing doses of AOH (50, 100, 300, 1000 and 2000 mg/kg body weight) and daily observed for clinical signs for one week. AOH was suspended in corn oil and the application volume was 10 mL/kg. Body weights were recorded daily. In preparation for the genotoxicity study, the limit dose of 2000 mg/kg was then repeatedly applied at 0, 24 and 45 h to one male and one female mouse.

7. Toxicokinetics of radiolabelled Alternariol in mice

7.1. Animal model

Male (= default sex for this study type) NMRI mice, approximately 7-8 weeks of age, were purchased from Charles River. NMRI mice are recognized by international guidelines as a recommended test system (e.g. EPA, OECD, EEC). For a period of 9 days prior to the start of the study, the mice were trained to become accustomed to handling. At the start of the study, the age of the mice was approx. 8-9 weeks. A total of 20 mice were used in this study (including 4 sentinel animals). The study was divided into two parts (see Table 4).

7.2. Treatment of animals

Treatment was done following overnight fasting (ca. 16 h) as a single oral dose by gavage. 10 ml/kg BW of the suspension consisting of [6-^{14}C] Alternariol and non-labelled Alternariol in corn oil was administered. The animals were given free access to food and water after administration.

7.3. Dose selection

The dose range finding study revealed that mice tolerated doses up to 2000 mg/kg without any impairment of their general health status. As it is stipulated in OECD guideline 417 “Toxicokinetics” that a maximum dose of 1000 mg/kg should be used for test compounds with low toxicity, this dose was chosen as the high dose. 200 mg/kg were used as low dose as it was expected that this dose level would be high enough to allow for test item recovery in tissues and excreta. The total radioactivity dose per animal was 1.6 MBq.

7.4. Study groups

The study groups are given in Table 4.

Table 4 Study Groups

Group	Purpose	Sex	Animal-No.	Samples for Radioactivity measurements (^{14}C)	Part *
1	Excretion, Tissue distribution day 7 **Low Dose** **200 mg/kg**	m	1101, 1102, 1103, 1104	Faeces, urine, exhaled air, heart, lung, liver, spleen, stomach#, small intestine#, large intestine#, muscle, kidneys, testes, renal fat, brain, bone, blood	1
2	Blood kinetics, Tissue distribution 24 h **Low Dose** **200 mg/kg**	m	2101, 2102, 2103, 2104	Blood (2, 6 and 24 h), heart, lung, liver, spleen, stomach#, small intestine#, large intestine#, muscle, kidneys, testes, renal fat, brain, bone, blood	
3	Excretion, Tissue distribution day 7 **High Dose** **1000 mg/kg**	m	3101, 3102, 3103, 3104	Faeces, urine, exhaled air, heart, lung, liver, spleen, stomach#, small intestine#, large intestine#, muscle, kidneys, testes, renal fat, brain, bone, blood	2
4	Blood kinetics, Tissue distribution 24 h **High Dose** **1000 mg/kg**	m	4101, 4102, 4103, 4104	Blood (2, 6 and 24 h), heart, lung, liver, spleen, stomach#, small intestine#, large intestine#, muscle, kidneys, testes, renal fat, brain, bone, blood	

* The study was divided into two parts which were performed one after the other because the capacity for the collection of exhaled air was limited to 4 animals.
# Contents measured separately

7.5. Observations

All animals were observed once a day. Individual body weights were recorded to the nearest 0.1 g before treatment start and also before necropsy.

7.6. Collection of exhaled air

Exhaled air was collected in groups 1 and 3. Each metabolism cage was placed individually in a clear hermetically sealed chamber which was continuously flushed with air at a flow rate of about 1 litre/min by a vacuum pump. A scheme of the collection equipment is shown in Figure 3. ^{14}C-CO_2 was collected by passing the air through two gas washing bottles containing 300 ml 3-methoxypropylamine (MOP, CO_2-trapping solution) and 300 ml water. A third gas washing bottle containing 0.001 % bromthymolblue solution (300 ml) was used to detect any remaining CO_2 escaping bottles 1 and 2 (Surplus CO_2 would cause a colour change from blue to green).

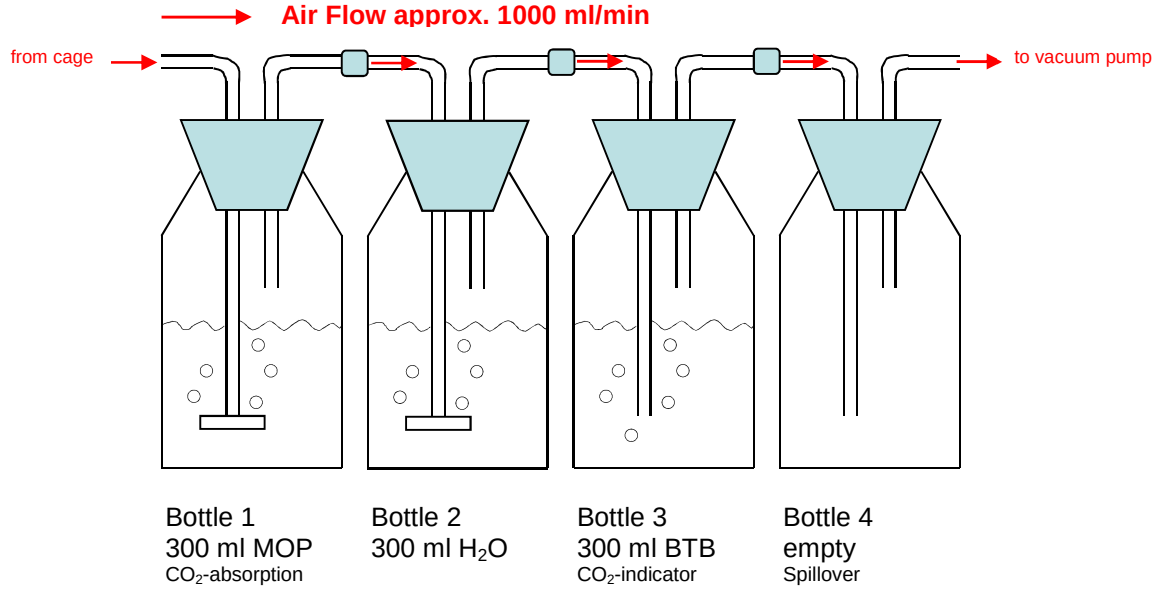


Figure 3 Collection of exhaled air. MOP: 3-methoxypropylamine, BTB: bromthymolblue

Samples were collected every 24 hours for a period of 7 days. All gas washing bottles were replaced by new ones with fresh solutions at every sampling time point (i.e. every 24 h). Furthermore, the colour of the bromthymolblue solution was checked and documented and the air flow was measured using a Defender 520 (Bios) and documented manually. Bottle 1 (3-methoxypropylamine) was placed in a plastic bowl filled with crushed ice to prevent evaporation of the CO_2-trapping solution. The crushed ice was changed twice daily throughout the 7-day sampling period.

7.7. Collection of urine and faeces

Urine and faeces were collected daily for 7 days in groups 1 and 3. Urine and faeces weights were documented on special data forms. After each collection of urine samples, the metabolism cages were washed with water to remove remaining urine and these washings were analyzed for radioactivity to ensure complete recovery of urinary excreted Alternariol.

7.8. Blood sampling

Blood samples were taken from the retroorbital vein plexus (group 2 and 4) at 2 and 6 h and from the *vena cava caudalis* after 24 h during necropsy.

7.9. Necropsy

Necropsy was done for all animals. The animals were anaesthetized by an overdose of CO_2. After collecting a blood sample in a Li-Heparin tube (ca. 200 µL), the animals were killed painlessly by cutting the *vena cava caudalis*. All animals were subjected to a complete necropsy, which included careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Organ weights of adrenals, brain, eyes, stomach, small intestine, large intestine, heart, kidneys, liver, lungs, spleen and testes were recorded to the nearest 0.1 g. The following organs and tissues were collected: heart, lung, liver, spleen, stomach, small intestine, large intestine, muscle, kidneys, testes, renal fat, brain, bone, blood.

7.10. Storage of samples

All organ, tissue, blood, excreta and cage wash samples were stored at –20 °C until analysis.

7.11. Radioactivity measurements

Analysis of blood, organ, tissue and excreta samples

Blood, organ, tissue and excreta samples were combusted using the sample oxidizer OX 500 (Zinsser Analytik, Frankfurt, Germany). Approximately 0.2 g of each blood, organ or tissue sample was used for one measurement. Faeces samples were homogenized in water 24 hours before analysis. About 0.2 g of faeces suspension was used. The oxidizer OX 500 converts the organic moiety of a sample to ^{14}C-carbon dioxide and water. ^{14}C-Carbon dioxide is sucked through special scintillator fluid (Oxysolve C 400, Zinsser Analytic Co., Frankfurt) trapping the radioactive gas completely.

Analysis of exhaled air

10 ml 3-methoxypropylamine (MOP) from bottle 1 were mixed with 10 ml Permaflour ® (Perkin Elmer) for scintillation counting. Water from bottle 2 was counted with Rotiszint ®. For this purpose, 5 ml water were combined with 15 ml Rotiszint.

Analysis of cage wash samples

About 0.2 g cage wash water was counted in 20 ml Rotiszint®.

7.12. Liquid scintillation counting

The ^{14}C-content of all blood, organ, tissue, faeces, urine, cage wash and exhaled air samples was quantified using a liquid scintillation counter (Beckman Coulter LS 6500). Each value was determined as a mean of two measurements.

Background correction

The limit of quantification (LOQ) was determined by replicate analysis of blank samples. During the analysis of study samples, 111 blank values obtained were counted and used to calculate the background for combusted samples. The LOQ for combusted samples was assumed to be 109 dpm (mean + 3SD). For samples analysed without combustion, background values were calculated from repeated analysis of the respective solvents and scintillation cocktails. 15 ml Rotiszint with 5 ml water were used as blank for cage wash samples and water from bottle 2 (62 dpm). In the case of MOP from bottle 1, 10 ml MOP and 10 ml Permafluor® were used for background correction (57 dpm). All activity data were corrected for the appropriate background values.

7.13. Quality assurance

The radioactive toxicokinetic study was performed under GLP. For this reason, the Quality Assurance Unit of Fraunhofer ITEM inspected critical phases of the study and audited the study plan, the raw

data, and the detailed GLP-conform final report, which will be available on request (tanja.hansen@item.fraunhofer.de).

8. Kinetics and metabolism of non-labelled Alternariol

8.1. Animal model

Male and female NMRI mice, approximately 5-6 weeks of age, were purchased from Charles River. The animals were acclimatized to the Fraunhofer ITEM laboratory conditions for 5 days. At the start of the study, the age of the mice was approx. 6-7 weeks. A total of 38 mice were used in this study (including 2 sentinel animals).

8.2. Treatment of animals

Oral treatment was done following overnight fasting (ca. 16 h) as a single oral dose by gavage. 10 ml/kg BW of the suspension of Alternariol in corn oil were administered. The animals were given free access to food and water after administration.

8.3. Dose selection

A previous radioactive toxicokinetic study (03N13517) was performed at 200 and 1000 mg/kg BW. Based on the results of this study, it was expected that the low dose level (200 mg/kg) would be high enough to allow for the recovery of AOH and potential metabolites in blood and urine. 200 mg/kg was thus selected as dose level for groups 1–2. Animals in group 3 received the limit dose used in the genotoxicity study 17G14017 (2000 mg/kg).

8.4. Study groups

The study groups are given in Table 5.

Table 5 Study groups in the non-radioactive kinetic study

Group	Purpose	Dose	Application	Animals	Samples
1	Excretion	Low dose 200 mg/kg	oral	5m/5f	Metabolism cage for 3 days. Collection of urine every 24 h
2	Blood Kinetics	Low dose 200 mg/kg	oral	12m/12f	Serial blood samples, 9 time points*, 3 samplings per animal (n=4)
3	Blood Kinetics/ metabolism	High dose 2000 mg/kg	oral	1m/1f	One blood sample at 2 h

Three additional animals from Study 17G14017 (group 2) also served for blood taking for analysis of AOH metabolites. These animals received the limit dose of 2000 mg/kg three times at 0, 24 and 45 h and were killed 48 h after the first application.

8.5. Collection of urine

Urine was collected daily for 3 days in group 1. Urine weights were documented on special data forms.

8.6. Blood sampling

Three blood samples were taken from each animal in K-EDTA tubes (Table 6). Two samples were taken from the retroorbital vein plexus (max 100 µL) and one from the *vena cava caudalis* during necropsy.

Table 6 Blood sampling schedule

Time (h)	Animal No.	Sampling Site
0.5	2101, 2102, 2103, 2104, 2201, 2202, 2203, 2204	Retroorbital Plexus
1	2105, 2106, 2107, 2108, 2205, 2206, 2207, 2208	Retroorbital Plexus
2	2109, 2110, 2111, 2112, 2209, 2210, 2211, 2212	Retroorbital Plexus
3	2101, 2102, 2103, 2104, 2201, 2202, 2203, 2204	Retroorbital Plexus
4	2105, 2106, 2107, 2108, 2205, 2206, 2207, 2208	Retroorbital Plexus
5	2109, 2110, 2111, 2112, 2209, 2210, 2211, 2212	Retroorbital Plexus
6	2101, 2102, 2103, 2104, 2201, 2202, 2203, 2204	Vena Cava
24	2105, 2106, 2107, 2108, 2205, 2206, 2207, 2208	Vena Cava
48	2109, 2110, 2111, 2112, 2209, 2210, 2211, 2212	Vena Cava

8.7. Necropsy

Necropsy was done for all animals. The animals were anaesthetized by an overdose of CO_2. A blood sample was collected in an EDTA tube (ca. 500 µL) and the animals were then killed painlessly by cutting the *vena cava caudalis*.

9. Pre-study *in vivo* alkaline comet assay

Due to the necessary change from the rat to the mouse model, based on limited amounts of the test item AOH, the *in vivo* alkaline comet assay had to be adapted to the smaller species, concerning single cell isolation from liver, stomach, and gut. In addition, an appropriate positive control with respective dose and treatment schedule had to be established for male NMRI mice.

9.1. Animal model

Male (= more prone to DNA damage than female animals) NMRI mice, approximately 10-12 weeks old were purchased from Charles River Deutschland (Sulzfeld, Germany). NMRI mice are the preferred species and strain for the mammalian erythrocyte micronucleus tests at Fraunhofer ITEM, with availability of profound historical data. As the main experiment for the *in vivo* comet assay with AOH was performed in parallel to the erythrocyte micronucleus assay, male NMRI mice were also used for the comet assay pre-study. A total of 12 mice were used in the pre-study (6 negative control animals and 6 positive control animals). Animals were accustomed to the new environment for two weeks before administration of vehicle or positive control.

9.2. Treatment of animals

Animals were treated with vehicle (tap water) or ethyl methanesulfonate (EMS, in tap water) as a single oral dose by gavage, after overnight fasting (approximately for 16 h). Ten mL/kg body weight

of the vehicle or EMS solution was administered 4 h before sacrifice. The animals were given free access to food and water after administration.

9.3. Dose selection

For the pre-study a dose of 300 mg/kg body weight EMS was applied - this dose is known from the literature to be an effective positive control in the mouse model.

9.4. Necropsy

Both administration and sacrifice were done with two subsets of three animals per treatment and with one day in between to enable both slide preparation and electrophoresis before treating the second subset of animals. Necropsy was done 4 h after administration. The animals were anaesthetized with Narcoren® about 5 min before sacrifice. After collecting a blood sample in a Li-Heparin tube (ca. 200 µL), the animals were killed painlessly by cutting the *vena cava caudalis*. The stomach, a part of the gut, one lung and one liver lobe were prepared and transferred to ice-cold tissue buffer (730.8 mg EDTA, 100 ml HBSS without Ca^{2+} and Mg^{2+}, pH 7.5 + 10 % dimethyl sulfoxide directly before use).

Stomach
Before transfer to tissue buffer, the stomach was opened at the *Curvatura major* and was rinsed with water to remove potentially remaining food. The stomach was finally fixed on a clean underlay with the inner part of the stomach upside and the brown stomach wall was scraped 2-4 times with a Teflon cell scraper. The tissue was transferred to a reaction cup containing 200 µL of ice-cold tissue buffer and was further minced with a sharp scissors until the buffer became cloudy. The buffer was then resuspended, using a cut pipette tip. About 50 µL of the suspension were then transferred to an additional reaction cup and placed on ice.

Gut
About 5 cm of the small intestine were prepared by discarding the first centimetre directly after the stomach. The gut lumen was flushed with tissue buffer and fixed with needles on a clean underlay. The lumen was opened and the tissue fixed with the inside on top. The single cells were then prepared as described under 10.7.

Lung
From the inner part of the lung lobe a 2-4 mm^3 piece was cut and directly transferred to a reaction cup containing 200 µL ice-cold tissue buffer. The tissue piece was then minced and resuspended as described under 10.7.

Liver
A piece of about 2-4 mm^3 was cut from the inner part of one liver lobe and was directly transferred to a reaction cup with 200 µL of ice-cold tissue buffer. The tissue piece was then minced and resuspended as described under 10.7.

Peripheral blood
Heparinized blood was directly suspended in agarose (15-20 µL per 250 µL of low melting agarose) and placed on agarose pre-treated slides.

9.5. Slide preparation and comet assay

The alkaline version of the comet assay was used for detection of DNA damage The single-cell gel (SCG)/comet assay represents a test principle for identifying agents with genotoxic activity in mammalian cells and for further characterizing types of DNA damage. The assay is based on electrophoretic mobility of DNA fragments in agarose gels on slides. Evaluation unit is the single cell.

The alkaline version (pH >13) of the comet assay is able to detect DNA single- (SSB) and double-strand breaks (DSB), DNA-DNA and DNA-protein cross-links, alkali-labile sites (ALS) and SSBs associated with incomplete DNA excision repair. DNA damage is detected as DNA migrating out of the cell nucleus during single-cell electrophoresis, resembling a comet tail. Tail intensity (TI) and tail length (TL) are proportional to the number of DNA strand breaks.

In brief, in the pre-study, approximately 50 µL of the different cell suspensions were suspended in 250 µL pre-heated 0.75 % low melting agarose and 100 µL of these samples were subsequently applied to each of two agarose pre-coated slides per animal and organ. After addition of a second agarose layer, the slides were lysed overnight at 4 °C to liberate the DNA. DNA-unwinding and electrophoresis were then done on ice, in 4 °C cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). DNA was finally stained with ethidium bromide and analysed using the Comet assay III Software from Perceptive Instruments (Steeple Bumpstead, Haverhill, UK). All methodological steps from cell lysis on were performed under red light to avoid unspecific DNA-damage due to UV-irradiation. As main endpoint the TI of 50 nuclei per slide and 2 slides per animal was determined. The TI is a direct measure for the amount of damaged DNA that can be standardized among various studies. Nevertheless, TL and TM were also analysed but were not included in the figure. An increase in TI, TL, or TM is indicative for the occurrence of direct DNA-damage.

The group mean TI and standard error of the mean of the median values of 50 nuclei per slide and two slides per animal were calculated. Statistical differences between the treatment groups (negative control and 300 mg/kg body weight EMS) were calculated for every organ using the Mann-Whitney-U-Test. Statistical significance was postulated if $P \leq 0.05$.

10. Combined mammalian erythrocyte micronucleus test and *in vivo* alkaline comet assay with Alternariol in mice

10.1. Guidelines for the conduct of the study

The combined mammalian erythrocyte micronucleus test and *in vivo* mammalian alkaline comet assay study was conducted in compliance with the OECD Guideline for the Testing of Chemicals No. 474 (Genetic Toxicology: "Mammalian Erythrocyte Micronucleus Test"; July 21, 1997) and the Principles of Good Laboratory Practice (German Chemicals Law, §19a, Appendix 1, of August 28, 2013). The study considered both the OECD Guideline draft ("*In vivo* Mammalian Alkaline Comet Assay"; December 2013) and the "Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports" (EFSA, 2012). It followed the regulations of the German Animal Protection Law (Tierschutzgesetz, August 07, 2013) and the valid Standard Operating Procedures of Fraunhofer ITEM.

10.2. Animal model

Male (= more prone to DNA damage than female animals) NMRI mice (in total 38 animals, 5-7 weeks old at delivery and purchased from Charles River Deutschland, Sulzfeld, Germany) were used as model system for the main study. NMRI mice are the preferred species and strain for the mammalian erythrocyte micronucleus tests at Fraunhofer ITEM, with availability of profound historical data. The micronucleus assay and the *in vivo* alkaline comet assay with AOH were performed partially in parallel, in the same animals. Animals were housed individually at 22 ± 2.0 °C, 55 ± 15 % humidity and controlled 12 h light/dark cycle.

10.3. Reference items

Corn oil was used as vehicle for AOH and as negative control. Cyclophosphamide monohydrate (CP; CAS No.: 6055-19-2) was used as positive control for the micronucleus assay (60 mg/kg body weight, dissolved in tap water). CP was administered once orally, after overnight starving, in a volume of 10 mL/kg body weight 24 h before sacrifice. Ethyl methanesulfonate (EMS; CAS No.: 62-50-0) was

used as positive control for the *in vivo* alkaline comet assay (300 mg/kg body weight, dissolved in tap water directly before use because of limited stability in aqueous solution). The EMS dose was applied once orally at a volume of 10 ml/kg body weight, 4 h before sacrifice. After administration the animals got free access to food and water.

10.4. Dose levels

A previous dose range finding study (Fraunhofer ITEM Study No. 03N13517) revealed that both male and female mice tolerated doses up to 2000 mg/kg body weight of AOH without any impairment of their general health status. The animals survived repeated application of 2000 mg/kg body weight without demonstrating treatment related effects. Due to the limited amount of AOH, both tests (mammalian micronucleus test and *in vivo* alkaline comet assay) were performed as "limit" tests, with one dose only, to allow for repeated application.

10.5. Treatment of animals

Animals were accustomed to the Fraunhofer ITEM animal house for at least 5 days before administration of vehicle (corn oil), AOH (in corn oil) or the respective positive controls (in water). Animals were clinically observed daily and only animals in good health condition were included in the study. The mice were randomly assigned to the study groups (based on body weights), and were identified unambiguously. A unique individual 3-digit number was assigned to each animal (first digit = treatment group, second and third digit = animal number). All data collected from the animal were filed under the allocated number. After acclimatization, animals were treated in total three times orally with AOH (2000 mg/kg) or vehicle (corn oil) after overnight starving, using an application volume of 10 ml/kg body weight. Treatments were performed at time points 0, 24, and 45 h. The animals were given free access to food and water after administration. For application of the positive controls see 10.3.
Five male animals were used and analysed per treatment group for the micronucleus assay, whereas 13 (vehicle control) or 10 (AOH and EMS) male NMRI mice were used and analysed per treatment group in the *in vivo* alkaline comet assay to verify the disturbance of slide preparation by corn oil treatment of the vehicle control and AOH animals. For the *in vivo* alkaline comet assay more animals were analysed than originally planned. For original identity of treatment groups and animal numbers see Table 7. In groups 1, 2, and 4, three additional animals were included as backups for histopathology. In group 2 the three additional animals also served for blood taking for analysis of AOH metabolites (Fraunhofer ITEM Study No. 03N14528). Due to the problems with comet assay slide preparation from stomach and gut tissue of corn oil and AOH treated animals, the three backups for groups 1, 2, and 4 were also used for the comet assay. Furthermore, a repeat test was performed with five control animals, two AOH treated animals, and two EMS treated animals to verify the adverse effect of corn oil on slide preparation. Total number of animals thus finally amounted to 38 male NMRI mice.
Five male animals were used and analysed per treatment group for the micronucleus assay, as shown in Table 7.

Table 7 Study design of the combined micronucleus test and alkaline comet assay study

Group	Treatment	Application*	Preparation	Number of mice	Test
1	Corn oil, 10 ml/kg b.w.	0, 24, 45 h	48 h	5 + 3 males	MT, CA, HP
2	Alternariol, 2000 mg/kg b.w.	0, 24, 45 h	48 h	5 + 3 males	MT, CA, HP, M
3	CP, 60 mg/kg b.w.	24 h	48 h	5 + 0 males	MT
4	EMS, 300 mg/kg b.w.	44 h	48 h	5 + 3 males	CA, HP

MT = Micronucleus test; CA = Comet assay; HP = Histopathology; M = Analysis of AOH metabolites; * time after start (time point 0 h) of the corn oil and AOH applications.

10.6. Observations

Body weights were determined by use of electronic balances at the time of randomization, prior to first administration of the test/reference substances and before bone marrow preparation and tissue sampling for the alkaline comet assay and histopathology. The animals were observed in defined intervals, following test/reference item administration and clinical signs of reaction to treatment were recorded. For recording of the individual body weights, clinical observations, and histopathological findings the PROVANTIS™ software was used (Instem Life Science System Ltd., Walton Industrial Estate, Staffs, ST 15 OLT, UK, version 8.4.3.1 or higher).

10.7. Preparation of diverse samples

Animals were sacrificed by CO_2 exposure. Animals receiving AOH and animals of the vehicle control group were sacrificed 48 h after the first application (= time point 0 h). Animals of the EMS positive control group were sacrificed 4 h after application, and animals receiving CP as positive control for the micronucleus test were sacrificed 24 h after CP administration. Because of the repeated exposure regime, only one sampling time was used for the corn oil and AOH treated animals. Preparation times were considered by planning and performing test and reference item applications.

Preparation of bone marrow and bone marrow smears

At the time of necropsy one femur of each mouse was collected (except for group 4) and cleaned of the surrounding muscle tissue. Ends of the femur/s were cut off. The bone marrow was washed out with foetal calf serum (FCS) and transferred into a tube. The bone marrow in the tube was gently pulled up and down till a fine cell suspension was observed and was subsequently centrifuged. Most of the supernatant was discarded and the cell pellet was carefully re-suspended in a very small volume of FCS, resulting in about 2 drops of bone marrow cell suspension per animal. From this suspension two smears (A and B) were prepared on defatted slides. The smears were air-dried for 24 h and stained according to Pappenheim with May-Grünwald- and Giemsa-solution.

Preparation of single cell suspensions and slides for the *in vivo* mammalian alkaline comet assay and conduct of the comet assay

After preparation of the femur(s) for generation of bone marrow smear for the micronucleus assay, the stomach, part of the small intestine and one liver lobe were prepared and transferred to ice-cold tissue buffer (730.8 mg EDTA, 100 ml HBSS without Ca^{2+} and Mg^{2+}, pH 7.5, + 10 % dimethyl sulfoxide directly before use). Stomach and small intestine were used in the comet assay as “first site of contact organs” and the liver served as systemic organ with high metabolic activity.

Stomach: Before transfer to tissue buffer, the stomach was opened at the *Curvatura major* and rinsed with ice-cold tissue buffer to remove potentially remaining food. Stomach was then transferred for some minutes to a container with ice-cold tissue buffer, before it was finally fixed on a clean underlay with the inner part of the stomach upside. After cutting of a small stripe for parallel histopathology, the brown stomach wall was then scraped 2-4 times with a Teflon cell scraper and the resulting tissue was transferred to a reaction cup containing 200 µL of ice-cold tissue buffer. The tissue was then further minced with sharp scissors until the buffer became cloudy. The resulting cell suspension was subsequently re-suspended using a pipette tip. Approximately 70 µL (depending on cell density) of the cell suspensions were finally transferred to additional reaction cups and placed on ice.

Small intestine: About 5 cm of the small intestine were prepared by discarding the first centimetre directly after the stomach and taking adjacent samples for histopathology. The gut lumen was then flushed with tissue buffer, put into a container with ice-cold tissue buffer for some minutes, before the sample was fixed with needles on a clean underlay. The lumen was subsequently opened and the tissue fixed with the inside facing upward. The single cells were then prepared as described under "Stomach" beginning with scraping 2-4 times with a Teflon cell scraper.

Liver: After a short period in ice-cold tissue buffer, a piece of about 2-4 mm^3 was cut from the inner part of one liver lobe and was directly transferred to a reaction cup with 200 µL of ice-cold tissue buffer. The tissue piece was then minced and resuspended as described under "Stomach". The rest of this liver lobe was used for histopathology.

The alkaline version of the comet assay was subsequently used for detection of local DNA damage. In brief, after single cell preparation approximately 70 µL of the different cell suspensions (depending on cell density) were suspended in at least 350 µL of pre-heated 0.75 % low melting agarose and thereof 100 µL were applied to each of three agarose pre-coated slides per animal and organ. After addition of a second agarose layer, the slides were lysed overnight at 4 °C to liberate the DNA. DNA-unwinding and electrophoresis were subsequently done on ice, using 4 °C cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). DNA was finally stained with ethidium bromide and stored in a humid environment at 4 °C till microscopic analysis. All methodological steps from cell lysis on were performed under red light to avoid unspecific DNA-damage due to UV-irradiation.

Preparation of tissues for histopathology

Histopathology was performed to detected possible local cytotoxicity of AOH. Determination of local cytotoxicity thereby enables better interpretation of comet assay results. Bone marrow (from sternum), cecum, colon, duodenum, oesophagus, forestomach, gall bladder, glandular stomach, ileum, jejunum, liver, rectum, and macroscopically observed lesions were fixed in formalin and processed according to standard procedures. All other organs like brain, kidney, pancreas, spleen, and lung were preserved in formalin. Samples were taken from all animals of the study and also the repeat experiment. The backup animals as well as the AOH-treated animals of the repeat experiment were also used for blood taking (AOH-treated animals) for analysis of AOH metabolites (Fraunhofer ITEM Study No. 03N14528).

10.8. Analyses

Microscopic analysis of bone marrow smears

The bone marrow smears were coded prior to microscopic analysis and were analysed by light microscopy under 630-1000x magnification. The incidence of micronucleated cells per 2000 polychromatic erythrocytes (PCE) was determined for each treated animal. The ratio of PCE to normochromatic erythrocytes (NCE) was calculated (PCE : NCE) by counting the number of PCE per 200 red blood cells. For both endpoints one of the two existing (A and B) bone marrow smears was

used. Five animals per treatment group, with the animal numbers 101-105, 201-205, and 301-305 were analysed.

Analysis of the comet assay slides

The comet assay slides were analysed using the Comet assay III Software from Perceptive Instruments (Steeple Bumpstead, Haverhill, UK). As the main endpoint the median TI of at least 50 nuclei per slide and three slides per organ and animal (150 nuclei per sample) were determined by excluding so-called "hedgehogs" (small or not-existing head and large diffuse tails). The TI is a direct measure for the amount of damaged DNA that can be standardized among various studies. Nevertheless, tail length (TL) and tail moment (TM) were also analysed and documented in parallel, but were not statistically evaluated and included in potential figures. An increase in TI/TL/TM on the slides is indicative for the occurrence of direct DNA-damage. The TI group means ± standard deviation of the TI means of the median values of 50 nuclei per slide and three slides per animal were calculated. Statistical differences between the treatment groups (vehicle control, positive control, AOH treated animals) were calculated for every of the three organs using the Mann-Whitney-U-Test. Statistical significance was postulated if $P \leq 0.05$. In addition, percentage of "hedgehogs" was determined for the at least 150 nuclei per sample, without documenting TI for these cells.

Histopathology

The samples for histopathology were evaluated, especially for signs of inflammatory or cytotoxic processes such as cell infiltration or degenerative, apoptotic and/or necrotic changes.

10.9. Evaluation of the results

Mammalian erythrocyte micronucleus test:

A micronucleus assay is judged as valid, if the vehicle controls demonstrate low spontaneous frequencies of micronucleus induction and if the positive control animals demonstrate significantly higher frequencies of micronucleus induction, as compared to the vehicle control animals. In addition, in test item (AOH) or reference item (CP) treated animals, the PCE ratio should not fall below 20 % of the vehicle control (corn oil) values to avoid unspecific effects due to excessive cytotoxicity in the bone marrow.

A mutagenic effect is claimed, if there is a clear test item related increase in the number of micronucleated cells, as compared to the respective vehicle controls. But, biological relevance of the results is considered first. A test item for which the test results do not meet the above criterion is considered non-mutagenic in this test.

As statistical method the U-test according to Mann-Whitney (treatment groups and positive control groups versus vehicle control) for two-group comparisons was used as an aid in evaluating the test results. Differences between group means were judged as statistically significant if $P \leq 0.05$.

In vivo mammalian alkaline comet assay

A comet assay is judged as valid if the concurrent data for the vehicle control animals are considered acceptable for addition to the laboratory historical negative control database, and the positive control induces a statistically significant increase in TI, as compared to the concurrent vehicle control animals, and are compatible with the historical positive control data. In addition, analysis of a sufficient number of appropriate cell nuclei (≥ 150) should have been possible.

Providing that all acceptability criteria are fulfilled, the test item AOH would be claimed positive if the limit dose of 3 x 2000 mg/kg body weight let to a statistically significant increase in TI, as compared to the concurrent vehicle control animals, and all results are outside of the historical negative control data for the respective tissue. AOH is then considered to induce DNA-strand breaks

in the tissue studied. Providing that the positive criteria are not met, AOH would be claimed negative and unable to induce DNA-strand breaks in the tissues studied.

If neither a clearly positive nor a clearly negative result can be claimed, the test item AOH is judged as equivocal. Due to the limited amount of the test items, comprehensive repeat assays or further investigations could not be performed with the given batch of the test item to clarify an unclear result. In the case of clearly positive or equivocal results, histopathology data are consulted for better data interpretation.

10.10. Quality Assurance

The combined micronucleus and comet assay study was performed under GLP. For this reason, the Quality Assurance Unit of Fraunhofer ITEM inspected critical phases of the study and audited the GLP-conform study plan, the raw data, and the detailed GLP-conform final report, which will be available on request (christina.ziemann@item.fraunhofer.de).

RESULTS

11. Analytical method development and validation (LC-MS/GC-MS)

The urine samples could not be satisfactorily addressed by the HPLC method. Matrix and blank values complicated in particular the analysis of the AOH metabolites. This made the implementation and validation of an additional GC-MS method inevitable. The validation reports of both methods are included in Appendix H as a separate report.

12. Alternariol purification

Due to the high demand of purified AOH a second batch was synthesized by the work group of Prof. Podlech (University, Karlsruhe). After delivery in October 2013 the whole purification procedure was started at Fraunhofer ITEM and was completed in December 2013. However, the yield and quality were not comparable with the previous batch. Brownish oxidized by-products required additional purification steps. Since the end of January 2014 in total 2.7 g highly purified AOH were available.

12.1. Confirmation of identity and purity of the processed and pooled AOH (NMR)

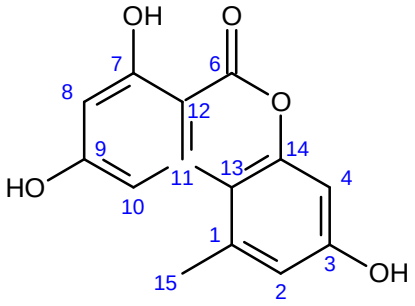
All purified AOH stocks were pooled into a single sample with a total weight of 3.40 g. Purity was determined by NMR spectroscopy against maleic acid (99.7 %) as an internal standard. The calculation was accomplished with the integral of the maleic acid signal at 6.18 ppm. The integral was 3.502.

Hence the AOH content was determined to be **93.4 ± 0.7 % (NMR)**.

The corresponding integrals (Ix) of AOH as well as the measured ^{1}H and ^{13}C NMR chemical shift [δ] are given in Table 8.

Table 8 ^{1}H and ^{13}C NMR chemical shifts [δ] of purified AOH measured in $CDCl_3$/DMSO-D_6 (50:50)

^{1}H-No.	AOH	Ix
2	6.63	1.010
4	6.58	1.007
8	6.31	
10	7.18	1.001
OH 3	10.05	
OH 7	11.75	0.998
OH 9	10.59	
CH3 15	2.69	3.000
^{13}C-No.		
1	138.0	
2	117.5	
3	158.3	
4	101.8	
6	165.3	
7	164.2	
8	101.0	
9	165.2	
10	104.4	
11	138.3	
12	97.5	
13	109.2	
14	152.7	
CH3 15	25.5	



13. Identity and HPLC peak confirmation of the oxidized metabolites of AOH (NMR/MS)

The intended method validation and the NMR spectroscopic structural confirmation of oxidized metabolites require a consistent separation (HPLC) and enrichment (peak cutting) of the components generated by the microsomal incubation.

13.1. HPLC separation and peak assignment

HPLC separation was carried out according to the literature specifications (Pfeiffer et al.; 2007). The numbering of the oxidized metabolite has been adjusted accordingly. Figure 4 shows different HPLC profiles of the extract from the incubation of AOH with NADPH-fortified rat liver microsomes. Distribution and intensity of all constituents are consistent with the specifications from the literature (Table 9 and Figure 4). Based on this separation method, the HPLC-cuts for the structural elucidation were performed. In addition, this HPLC method is subject of the analytical method validation.

Table 9 Fit of the retention times between Fh-ITEM and the corresponding data from the literature (Pfeiffer et al., 2007)

Peak No.	Retention time [min]		Quotient
	Fh-ITEM	Literature	Lit. / ITEM
1	10.5	12.3	1.174
2	11.3	12.8	1.136
3	11.9	13.4	1.124
4	12.8	13.8	1.076
5	14.7	15.3	1.041

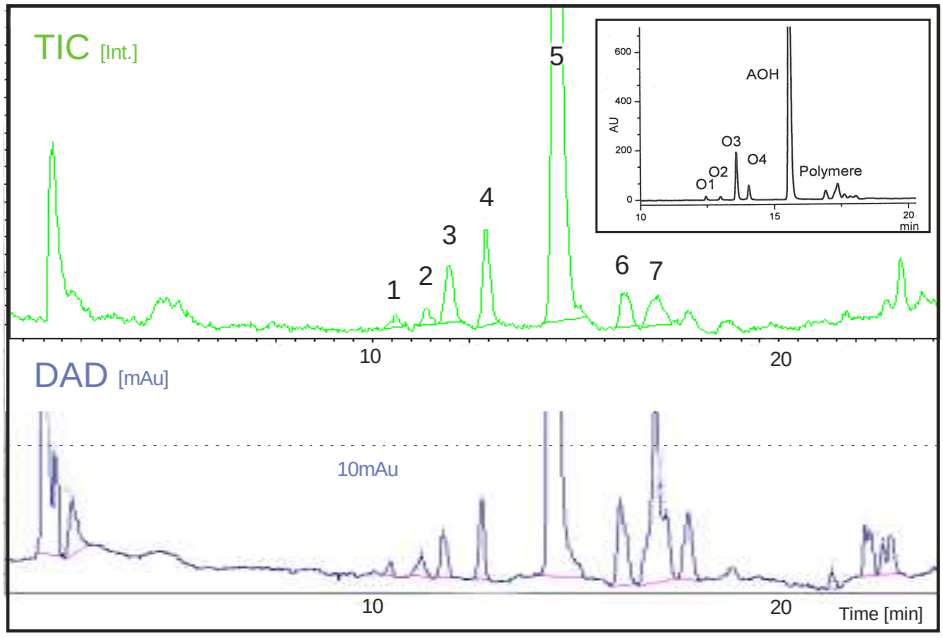


Figure 4 Established liquid chromatography method for Alternariol (AOH) separation at Fraunhofer ITEM. TIC[7] and DAD[8] chromatogram profiles of the microsomal AOH incubation are shown. The separation of AOH and the 4 oxidized metabolites was carried out according to the literature specification (Box on the right, Pfeiffer et al., 2007). The Peak numbering given in TIC corresponds to the literature specification (Peak 1 = O1, etc.).

13.2. Enrichment of the oxidized metabolites of AOH (HPLC)

The extraction of the AOH incubation with ethyl acetate resulted in an approximately 150-fold enrichment. This starting solution was chromatographed repeatedly under the given conditions and the selected peaks were cut. The pooled cut volumes were then evaporated to dryness in a nitrogen stream and resolved in the respective solvent for the NMR or MS analyses.

[7] TIC = total ion chromatogram.
[8] DAD = diode array detector.

13.3. General principal of the NMR data interpretation

All chemical shifts of AOH and its molecular structure are summarized in Table 8. These NMR chemical shifts have been used as reference for the unaltered chemical structure of AOH. In the following comparative data presentation, the chemical shifts of structurally identical counterparts of the oxidized metabolites investigated here are printed in black and those molecule parts that are different or include significant alterations are highlighted in red. All corresponding NMR spectra are listed in Appendix A. Furthermore, a comprehensive graphical representation of all cross-peaks in the 2D NMR spectra has been omitted for clarity. In most cases a tabular form has been used instead (Appendix C).

13.4. General principal of the MS data interpretation

The MS spectra reveal primarily the molecular weight of the oxidized metabolites investigated (Appendix B). Note, this would be by the use of NMR spectroscopy alone not possible. All the prominent MS^2 fragments are subjected to further fragmentation (MS^3). It is then only verified whether the resulting fragmentation pattern is compliant with the initial molecular structure. Statements regarding the exact position of additional hydroxyl-groups are not generally possible and the structural information from NMR spectroscopy must be used instead.

13.5. Structural confirmation of the metabolite O1 at peak 1 (RT = 10.48 min)

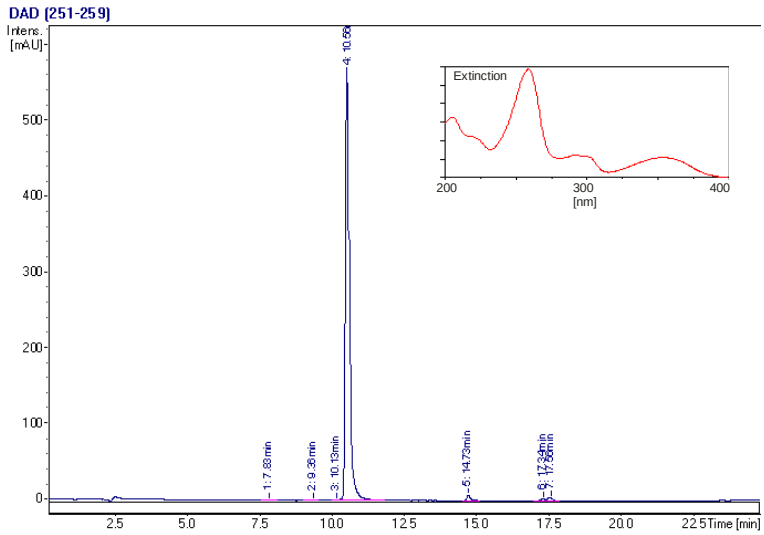
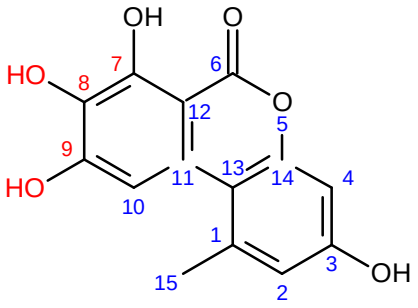


Figure 5 HPLC-UV-Chromatogram and UV spectrum of the purified metabolite O1 at peak 1 (RT=10.5min), purity approx. 97 %

The HPLC-UV chromatogram and the UV spectrum of the purified peak 1 (O1) are shown in Figure 5. ^{1}H NMR spectra are shown in Appendix A and the corresponding mass spectrometric data in Appendix B.The corresponding NMR data is given in Table 10. The assignments of the signals made here are based mainly on the results of two-dimensional NMR spectroscopy. A tabular form of all observed cross peaks in COSY, HSQC and HMBC 2D-spectra are listed in Tables 25 and 26 in Appendix C.

Table 10 Comparison of ^{1}H and ^{13}C NMR chemical shifts [δ] of AOH and 8-Hydroxy-AOH (Peak 1/O1) measured in $CDCl_3$/DMSO-D_6 (50:50)

^{1}H-No.	AOH	8-Hydroxy-AOH	delta
2	6.63	6.62	-0.01
4	6.58	6.59	0.01
8	6.31		-6.31
10	7.18	7.30	0.12
OH 3	10.05	9.83	-0.22
OH 7	11.75	11.74	-0.01
OH 8		8.83	8.83
OH 9	10.59	10.15	-0.44
CH3 15	2.69	2.69	0.00
^{13}C-No.			
1	138.0	126.9	-11.1
2	117.5	117.3	-0.2
3	158.3	157.2	-1.1
4	101.8	101.7	-0.1
6	165.3		
7	164.2	150.8	-13.4
8	101.0	131.2	30.2
9	165.2	153.7	-11.5
10	104.4	104.4	0.0
11	138.3		
12	97.5	98.2	0.7
13	109.2	109.5	0.3
14	152.7	151.9	-0.8
CH3 15	25.5	25.3	-0.2



Conclusion: All analytical data are consistent with the given structure of metabolite O1 at peak 1. A detailed discussion of all aspects of the signal assignments made is omitted here. However, the 8-position of the additional hydroxyl group can be surely confirmed.

13.6. Structural confirmation of the metabolite O2 at peak 2 (RT = 11.27 min)

The HPLC-UV chromatogram and the UV spectrum of the purified peak 2 are shown in Figure 6. ^{1}H NMR spectra are shown in Appendix A and the corresponding mass spectrometric data in Appendix B.The corresponding NMR data is given in Table 11. The assignments of the signals made here are based mainly on the results of two-dimensional NMR spectroscopy. A tabular form of all observed cross peaks in COSY, HSQC and HMBC 2D-spectra are listed in Tables 28 and 29 in Appendix C.

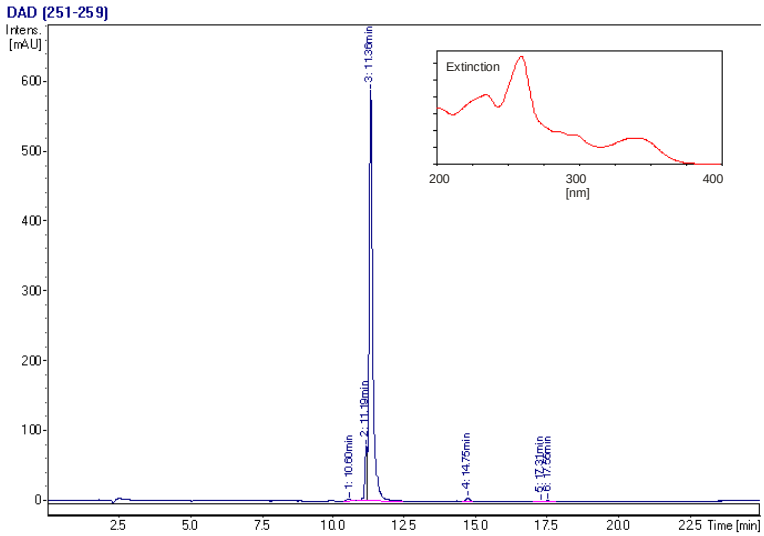
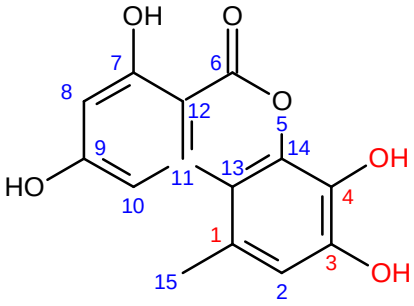


Figure 6 HPLC-UV-Chromatogram and UV spectrum of the purified metabolite O2 at peak 2 (RT=11.3min), purity approx. 90 %

Table 11 Comparison of ^{1}H and ^{13}C NMR chemical shifts [δ] of AOH and 4-Hydroxy-AOH (Peak 2 / O2) measured in $CDCl_3$/DMSO-D_6 (50:50)

^{1}H-No.	AOH	4-Hydroxy-AOH	delta
2	6,63	6,65	0,02
4	6,58		-6,58
8	6,31	6,32	0,01
10	7,18	7,20	0,02
OH 3	10,05	9,48	-0,57
OH 4		8,92	8,92
OH 7	11,75	11,83	0,08
OH 9	10,59	10,52	-0,07
CH3 15	2,69	2,63	-0,06
^{13}C-No.			
1	138,0	126,2	-11,8
2	117,5	116,8	-0,7
3	158,3	146,6	-11,7
4	101,8	131,1	29,3
6	165,3		
7	164,2	164,2	0,0
8	101,0	100,9	-0,1
9	165,2	165,5	0,3
10	104,4	104,5	0,1
11	138,3		
12	97,5	97,4	-0,1
13	109,2	109,5	0,3
14	152,7		
CH3 15	25,5	24,9	-0,6



Conclusion: All analytical data are consistent with the given structure of metabolite O2 at peak 2. A detailed discussion of all aspects of the signal assignments made is omitted here. However, the 4-position of the additional hydroxyl group can be surely confirmed.

13.7. Structural confirmation of the metabolite O3 at peak 3 (RT = 11.92 min)

The HPLC-UV chromatogram and the UV spectrum of the purified peak 3 are shown in Figure 7. ^{1}H NMR spectra are shown in Appendix A and the corresponding mass spectrometric data in Appendix B.The corresponding NMR data is given in Table 12. The assignments of the signals made here are based mainly on the results of two-dimensional NMR spectroscopy. A tabular form of all observed cross peaks in COSY, HSQC and HMBC 2D-spectra are listed in Tables 31 and 32 in Appendix C.

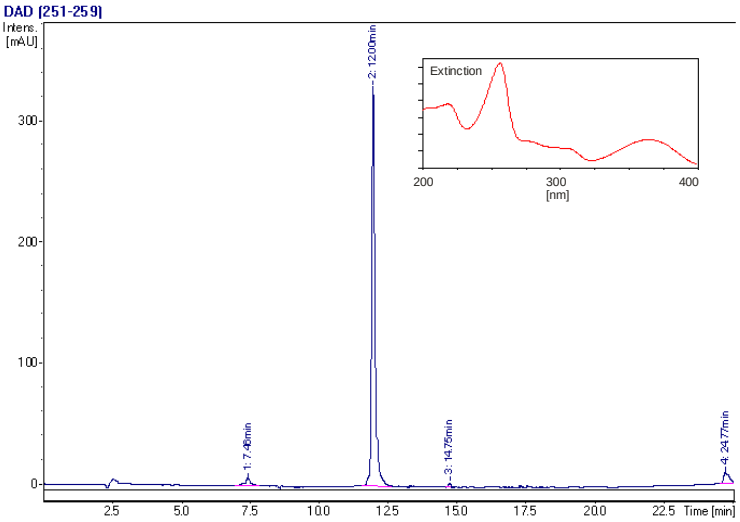
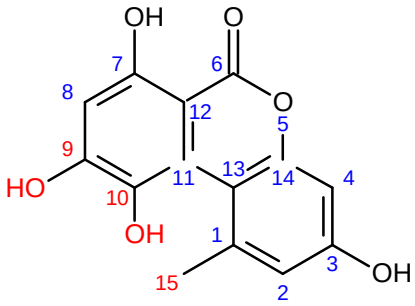


Figure 7 HPLC-UV-Chromatogram and UV spectrum of the purified metabolite O3 at peak 3 (RT=11.9 min), purity approx. 93 %

Table 12 Comparison of ^{1}H and ^{13}C NMR chemical shifts [δ] of AOH and 10-Hydroxy-AOH (Peak 3/O3) measured in $CDCl_3$/DMSO-D_6 (50:50)

^{1}H-No.	AOH	10-Hydroxy-AOH	delta
2	6,63	6,52	-0,11
4	6,58	6,46	-0,12
8	6,31	6,30	-0,01
10	7,18		
OH 3	10,05	9,60	-0,45
OH 7	11,75	11,46	-0,29
OH 9	10,59	? 6,33	
OH 10		? 6,33	
CH3 15	2,69	2,48	-0,21
^{13}C-No.			
1	138,0	138,9	0,9
2	117,5	115,3	-2,2
3	158,3		
4	101,8	100,1	-1,7
6	165,3		
7	164,2	158,9	-5,3
8	101,0	96,9	-4,1
9	165,2		
10	104,4	139,0	34,6
11	138,3		
12	97,5	95,3	-2,2
13	109,2		
14	152,7		
CH3 15	25,5	24,0	-1,5



Conclusion: All analytical data are consistent with the given structure of the metabolite O3 at peak 3. A detailed discussion of all aspects of the signal assignments made is omitted here. However, the 10-position of the additional hydroxyl group can be surely confirmed.

13.8. Structural confirmation of the metabolite O4 at peak 4 (RT = 12.82 min)

The HPLC-UV chromatogram and the UV spectrum of the purified peak 4 are shown in Figure 8. ^{1}H NMR spectra are shown in Appendix A and the corresponding mass spectrometric data in Appendix B.The corresponding NMR data is given in Table 13. The assignments of the signals made here are based mainly on the results of two-dimensional NMR spectroscopy. A tabular form of all observed cross peaks in COSY, HSQC and HMBC 2D-spectra are listed in Tables 34 and 35 in Appendix C.

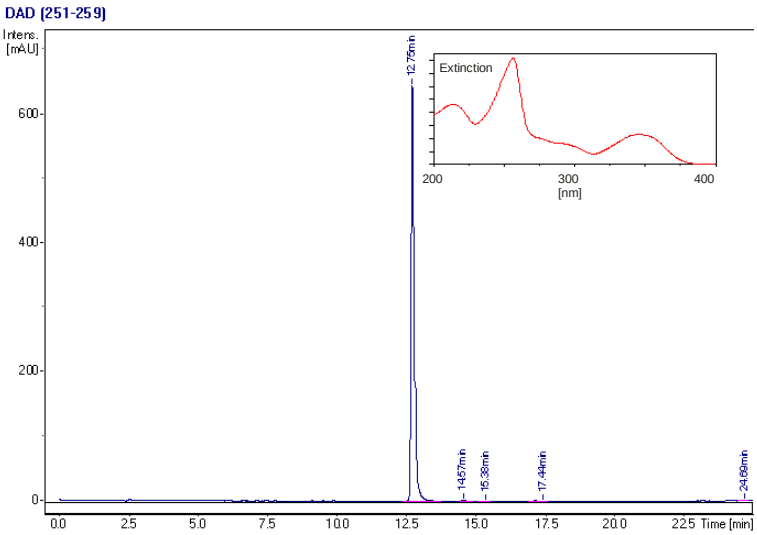


Figure 8 HPLC-UV-Chromatogram and UV spectrum of the purified metabolite O4 at peak 4 (RT=12.8 min), purity approx. 89 %

Conclusion: All analytical data are consistent with the given structure of metabolite O4 at peak 4. A detailed discussion of all aspects of the signal assignments made is omitted here. However, the 2-position of the additional hydroxyl group can be surely confirmed.

The assignments made in the literature of individual metabolites to the retention times have thus proven to be consistently accurate (Figure 9).

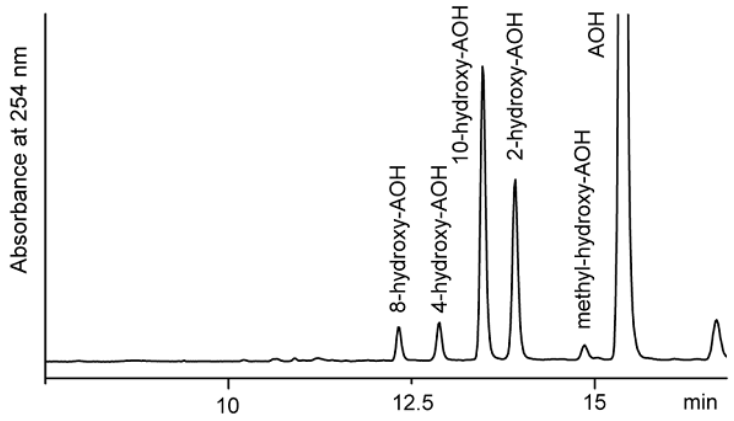
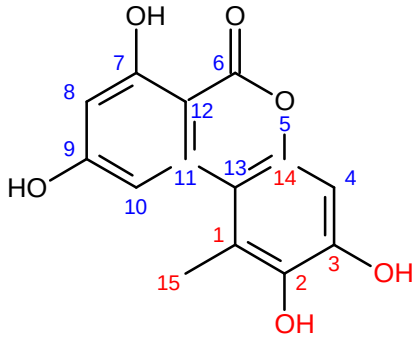


Figure 9 Assignments of the retention times of the oxidative metabolites O1-O4 according to Pfeiffer et al. (2007)

Table 13 Comparison of ^{1}H and ^{13}C NMR chemical shifts [δ] of AOH and 2-Hydroxy-AOH (Peak 4 / O4) measured in $CDCl_3$/DMSO-D_6 (50:50)

^{1}H-No.	AOH	2-Hydroxy-AOH	delta
2	6,63		-6,63
4	6,58	6,68	0,10
8	6,31	6,31	0,00
10	7,18	7,24	0,06
OH 2		8,23	8,23
OH 3	10,05	10,18	0,13
OH 7	11,75	11,85	0,10
OH 9	10,59	10,49	-0,10
CH3 15	2,69	2,60	-0,09
^{13}C-No.			
1	138,0	121,8	-16,2
2	117,5	141,5	24,0
3	158,3	147,2	-11,1
4	101,7	101,0	-0,7
6	164,9	nd	
7	164,3	164,4	0,1
8	100,9	100,8	-0,1
9	165,2	165,1	-0,1
10	104,3	104,4	0,1
11	138,2	nd	
12	97,5	98,0	0,5
13	109,2	109,4	0,2
14	152,7	145,1	-7,6
CH3 15	25,4	15,9	-9,5



14. Identity and GC peak confirmation of the oxidized metabolites of AOH (NMR/MS)

The gas chromatic separation of the trimethylsilylated AOH metabolites shows a different retention order in comparison with the HPLC separation. However, the assignment of the individual metabolites was successfully performed using the identified peak cut samples from the previous section for the GC-MS analysis. The peak sequence obtained is, furthermore, consistent with the results from the work of Schebb (2005). The four metabolites were monitored in GC-MS on the base ion *m/z* 547. Figure 10 shows the general formation of the base ion *m/z* 547 from the individual trimethylsilylated AOH metabolites (*m/z* 562) and Table 14 illustrates the assignments made for the obtained peak sequences in LC and GC.

Table 14 Mapping of the hydroxylated AOH metabolites with the peak sequence obtained from different HPLC and GC separations

HPLC-MS (Fh-ITEM)	GC-MS (Schebb 2005)	GC-MS (Fh-ITEM)
8-hydroxy-AOH 10.48 min	10-hydroxy-AOH 19.1 min	10-hydroxy-AOH 18.2 min
4-hydroxy-AOH 11.27 min	4-hydroxy-AOH 20.3 min	4-hydroxy-AOH 19.4 min
10-hydroxy-AOH 11.92 min	8-hydroxy-AOH 21.4 min	8-hydroxy-AOH 20.2 min
2-hydroxy-AOH 12.82 min	2-hydroxy-AOH 22.6 min	2-hydroxy-AOH 21.3 min

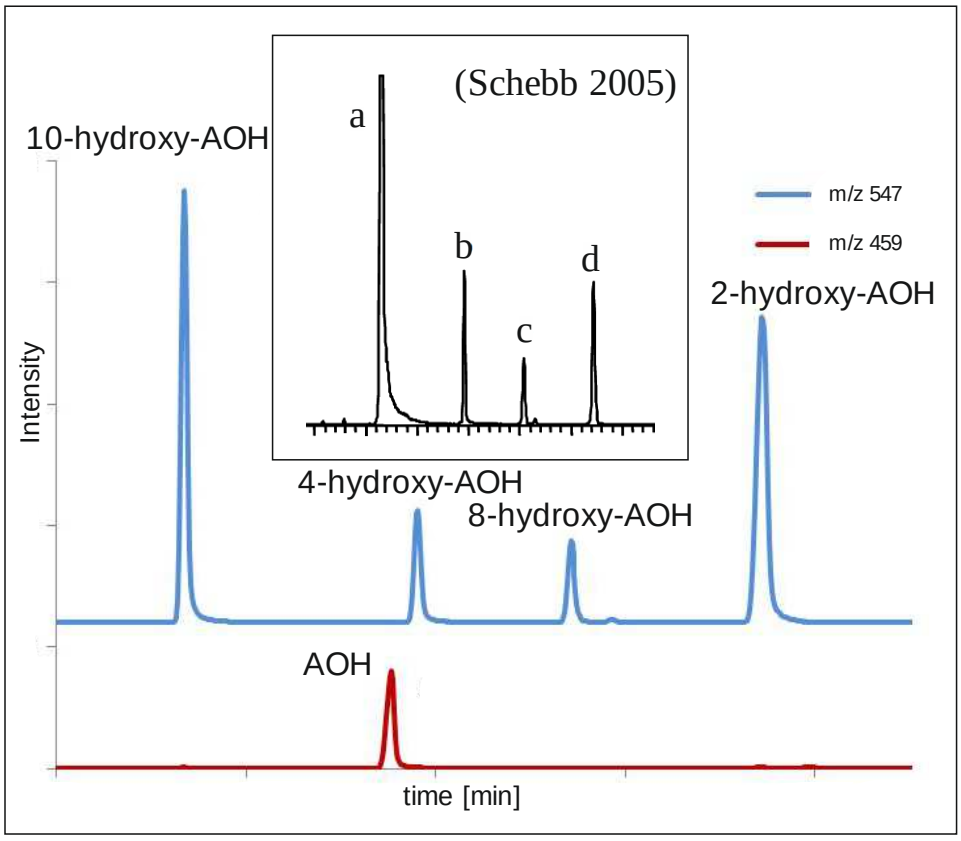


Figure 10 Gas chromatograms of AOH and its four hydroxyl metabolites traces in rat microsome extract. AOH monitored on *m/z* 459. Metabolites monitored on *m/z* 547. All assignments made are approved by references made from the corresponding HPLC peak cuts. Box on top: Comparison with similar results of Schebb (2005).

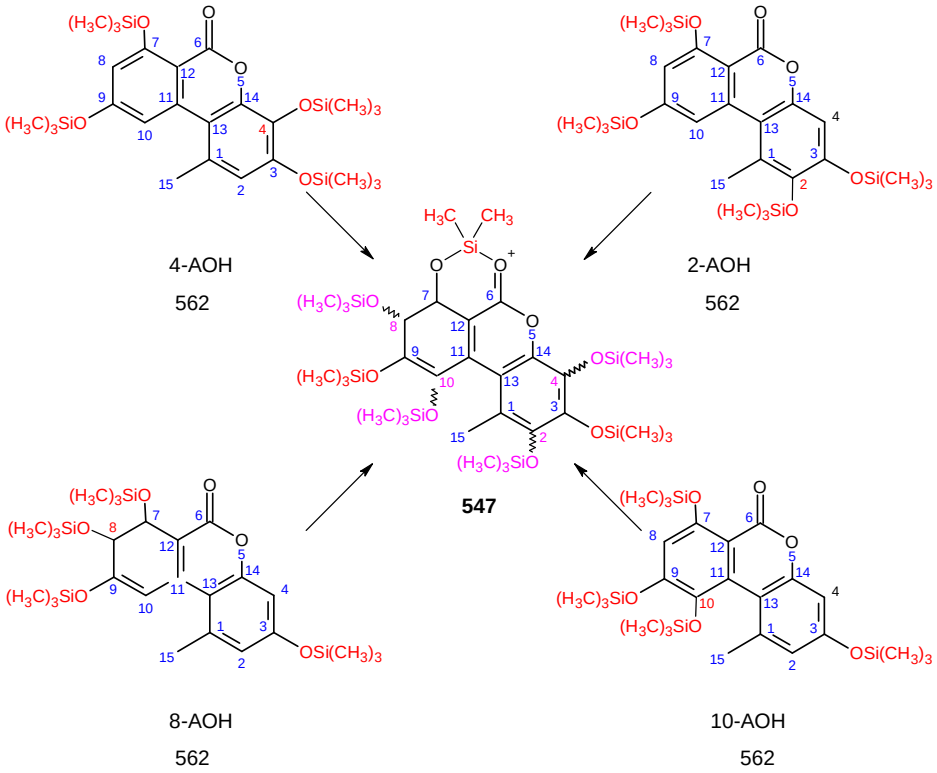


Figure 11 Illustration of the EI-MS triggered formation of the base ion *m/z* 547 (100%) from the four trimethylsilylated oxidative metabolites. The base ion represents the $[M-15]^+$ ion of monohydroxylated AOH independent of the individual OH binding site. However, all possible trimethylsilylated OH binding sites are marked in purple for the ion m/z 557. Note in reality it is only one trimethylsilylated OH group for each represented metabolite implemented.

15. Results of the dose range finding study

AOH proved to be nontoxic at doses up to 2000 mg/kg, even after repeated application. Body weight data are given in Table 15.

Table 15 Body weight data (g) of the dose range finding study

Dose mg/kg	sex	Day 0 (Treatment)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
50	male	36.8	38.1	38.3	38.9	39.9	39.6	38.6	38.3
50	female	31.1	30.7	29.8	30.4	30.9	31.4	31.7	30.0
100	male	43.1	44.1	44.4	45.0	44.1	44.7	44.6	43.7
100	female	32.4	32.0	31.8	31.0	31.8	31.3	31.1	31.0
300	male	39.9	39.4	39.3	40.4	40.0	41.0	39.3	39.4
300	female	35.9	35.7	35.3	34.3	35.5	36.1	35.8	35.9
1000	male	41.1	40.3	39.6	40.2	38.7	39.2	39.2	38.9
1000	female	30.4	30.3	30.7	31.6	31.6	31.7	31.9	31.1
2000	male	45.6	46.9	46.8	46.3	46.2	45.8		
2000	female	33.4	33.3	34.1	33.1	33.4	33.3		
		Treatment on three consecutive days							
2000	male	40.1	39.5	39.5					
2000	female	34.1	34.5	33.6					

16. Results of the radioactive toxicokinetic study

16.1. Animal data

Survival of animals and general health status

After administration of the test item, the mice showed a normal general health status. No effect of the administration of Alternariol on the general health status was observed during the 7-day observation period.

Body weights

Body weight data are presented in Table 16. During the randomization process, the animals with the greatest deviation from the group mean were rejected. A slight weight loss was observed in the animals used in the excretion studies (Groups 1 and 3). This effect is not considered treatment related but due to the housing in metabolism cages for one week.

Table 16 Body weight data (g)

Animal-No	Day of Treatment	Day of Necropsy (24 h)	Day of Necropsy (day 7)
1101	35.8	-	32.6
1102	36.5	-	35.1
1103	39.1	-	33.4
1104	38.6	-	33.4
2101	38.2	38.6	-
2102	35.9	38.9	-
2103	39.9	52.7	-
2104	37.2	50.7	-
3101	38.8	-	33.7
3102	37.2	-	39.0
3103	37.6	-	37.5
3104	37.0	-	33.4
4101	38.2	40.9	-
4102	37.8	40.3	-
4103	38.0	39.3	-
4104	37.0	37.0	-

Observations upon necropsy

Gross pathology upon necropsy of animals did not show any treatment-related effects. Individual organ weights were within the normal range for this species, sex and age.

16.2. Radioactivity measurements

Radioactivity dose

Based on the actual radioactivity determined in the application solution, the total radioactivity dose per animal was calculated. A mean radioactivity of 1.6 MBq was applied per animal and this value was used for all calculations.

Excretion of Alternariol

84.5 ± 6.4 % (low dose) and 90.7 ± 4.3 % (high dose) of the total ^{14}C activity were excreted via faeces during the 7 day collection period. The main proportion was excreted during the first 24 h. Urinary excretion (i.e. activity recovered in urine and cage wash samples) of ^{14}C accounted for 9.3 ± 1.3 % (low dose) and 5.7 ± 0.7 % (high dose) of the total dose applied. Again, the main proportion was excreted during the first 24 h collection interval. Only marginal ^{14}C activities were recovered in exhaled air, the levels being 0.05 ± 0.01 % and 0.03 ± 0.01 % in the low and high dose group, respectively.

Blood kinetics

Analysis of radioactive Alternariol in blood revealed, that the proportion of the dose administered, which was recovered in blood did not exceed 0.06 % during the first 24 hours after administration.

Tissue distribution

After 24 h 0.78 ± 0.4 % and 0.12 ± 0.1 % of the dose applied was found in the low and the high dose group, respectively. Highest radioactivity levels were detected in the gastrointestinal tract accounting for 98 % of the dose recovered. At the 7 day timepoint, only trace amounts of radioactivity were detected.

Mass balance recovery

Mass balance recoveries obtained after 7 days were satisfactory and accounted for 94 ± 6 % in the low dose group and 97 ± 4 % in the high dose group (Table 17).

Table 17: Mass balance recoveries 7 days after oral administration of ^{14}C-Alternariol to mice. (% of total dose applied)

Animal No	Dose	Faeces	Urine	Exhaled Air	Organs / Tissues	Total
1101	Low Dose (200 mg/kg)	78.81	8.54	0.043	0.002	87.40
1102		78.28	11.10	0.042	0.004	89.43
1103		87.40	9.76	0.058	0.002	97.22
1104		93.55	7.79	0.068	0.003	101.41
Mean		**84.51**	**9.30**	**0.05**	**0.00**	**93.86**
SD		6.35	1.26	0.01	0.00	5.70
3101	High Dose (1000 mg/kg)	90.86	6.35	0.030	0.140	97.38
3102		86.64	6.42	0.032	0.290	93.38
3103		97.67	4.72	0.032	0.041	102.46
3104		87.70	5.14	0.019	0.008	92.87
Mean		**90.72**	**5.66**	**0.03**	**0.12**	**96.52**
SD		4.30	0.74	0.01	0.11	3.85

* heart, lung, liver, spleen, stomach, small intestine, large intestine, muscle, kidneys, testes, adipose, brain, bone, blood

17. Results of the non-radioactive toxicokinetic study

17.1. Survival of animals and general health status

After administration of the test item, the mice showed a normal general health status. No effect of the administration of Alternariol on the general health status was observed during the 3-day observation period.

17.2. Blood kinetics of AOH

In male and female NMRI mice receiving a single oral dose of 200 mg/kg body weight, blood levels of AOH in the two-digit ng/mL range were detected during the first hours after application (see Table 18). After 6 h (males) and 24 h (females), AOH blood levels were below the limit of quantification. Individual results are shown in Appendix D.

Table 18 Blood levels of AOH as determined by HPLC-MS after oral application of 200 mg/mL AOH to NMRI mice

Time (h)	MALES		FEMALES	
	Mean AOH (ng/mL)	STDEV	Mean AOH (ng/mL)	STDEV
0.5	53.0	7.53	90.2	58.6
1	40.3	10.3	43.0	25.4
2	66.2	34.1	33.3	19.1
3	23.1	7.09	35.3	12.8
4	13.7*	--	41.3*	--
5	14.7*	--	28.5	11.3
6	BLQ	--	27.1	--
24	BLQ	--	BLQ	--
48	BLQ	--	BLQ	--

* single value
BLQ – below limit of quantification (10.8 ng/mL)

Toxicokinetic parameters were calculated using the software Kinetica® using group mean values (Table 19). For these calculations, values below the LOQ were set to ½ LOQ (5.4 ng/mL). The AUC was computed using the Log Linear trapezoidal method.

Table 19 Toxicokinetic parameters calculated from blood levels after oral application of 200 mg/mL AOH to NMRI mice

Parameter	Unit	Males	Females
Cmax	ng/ml	66,2	90,2
Tmax	h	2	0.5
AUClast	(h)*(ng/ml)	158.566	370.133
Lz (Elimination Rate Constant)	1/h	0.611118	0.0757238
Thalf (Half-Life of Elimination)	h	1.13423	9.15363
MRT (Mean Residence Time)	h	2.34979	12.4518
Vz (Apparent Volume of Distribution)	mg/kg/(ng/ml)	1.95498	5.98303

C_{max} levels were comparable between males and females accounting for 66.2 and 90.2 ng/mL, respectively. T_{max} was reached after 2 h in males and after 0.5 h in females. Elimination seemed to occur slightly faster in males resulting in different $t_{½}$ estimations in males and females, the values being 1.13 h for males and 9.15 h for females.

17.3. Metabolism of AOH

Analysis of AOH metabolites was done in blood samples of mice receiving 2000 mg/kg AOH. Two animals, one male (3101) and one female (3201) received a single oral dose and blood was taken 2 h after application. Five additional animals were treated within the genotoxicity study with three repeated oral doses at 0, 24 and 45 h and blood was taken 3 h after the last treatment. Individual results are shown in Appendix F.

Three metabolites were found in blood, namely 4-hydroxy-AOH (O2), 10-hydroxy-AOH (O3) and 2-hydroxy-AOH (O4). Absolute concentrations of the metabolites could not be determined, as the limited availability of analytical standards did not allow for a sound calibration for each metabolite.

17.4. Urinary excretion of AOH and its metabolites

The excretion of AOH and its metabolites via urine was followed in 5 male and 5 female animals over 3 days. Group mean values for AOH are given in Table 20 and calculated values for the total excretion of AOH and its metabolites are given in Table 21. Individual results are shown in Appendix E. Four metabolites of AOH were detected in urine, namely 8-hydroxy-AOH (O1), 4-hydroxy-AOH (O2), 10-hydroxy-AOH (O3) and 2-hydroxy-AOH (O4). Absolute concentrations of the metabolites could not be determined with certainty as the limited availability of analytical standards did not allow for a sound calibration for each metabolite. However, calibrations of the metabolites were made from the left-over samples of the HPLC cuts. The calibrations do not meet the validation standard, but allow semi-quantitative statements in ng/mL.

Table 20 Excretion of AOH in urine. Results are ng excreted. Mean ± SD of 5 animals are shown

Males		**Females**	
Time (h)	AOH	Time (h)	AOH
24	5099 ± 7122	24	10438 ± 8646
48	6579 ± 5750	48	1804 ± 801
72	2060 ± 1671	72	136 ± 41

Table 21 Total excretion of AOH and its metabolites in urine during the 72 h collection period. Note, that only the AOH data are based on a validated calibration method, whereas the amounts of AOH metabolites are estimates

Animal	**AOH (ng)**	**10-OH AOH (ng)**	**4-OH AOH (ng)**	**8-OH AOH (ng)**	**2-OH AOH (ng)**	**Sum (ng)**	**% of total dose**
1101	23016	14	14	25	61	23131	0.394
1102	3111	5	6	0	11	3133	0.053
1103	16398	13	18	0	48	16478	0.280
1104	23975	143	38	669	147	24972	0.425
1105	2190	2	4	1	7	2205	0.038
1201	24109	27	18	0	49	24202	0.568
1202	6469	24	11	1	26	6530	0.153
1203	2554	13	6	0	13	2585	0.061
1204	8467	9	6	0	19	8501	0.199
1205	20098	65	12	90	36	20301	0.476

18. Results of the *in vivo* comet assay pre-study

In the *in vivo* alkaline comet assay pre-study six negative and six EMS-treated (300 mg/kg body weight, 4 h of oral exposure) animals were analysed concerning direct DNA-damage. The intention of the pre-study was to establish an appropriate positive control protocol and to establish preparation of single cell suspensions from stomach, gut, lung and liver of NMRI mice. In addition the pre-study aimed at generating first historical data. In the negative control animals highest group mean TI and thus the highest basal DNA-damage was observed for gut (8.7 ± 2.19 %), followed by stomach (2.0 ± 0.87 %), blood (0.1 ± 0.03 %), and liver (0.1 ± 0.02 %). EMS was demonstrated to significantly induce an increase in group mean TI in blood, liver, stomach, and gut of male NMRI mice by using the application protocol given above. Highest effects were observed in gut (group mean TI: 21.5 ± 4.39 %) followed by stomach (group mean TI: 6.1 ± 0.29 %), blood (group mean TI: 3.8 ± 0.78 %) and liver (group mean TI: 3.0 ± 0.45 %). The highest effects were thus obvious in the tissues of first contact (see Figure 12).

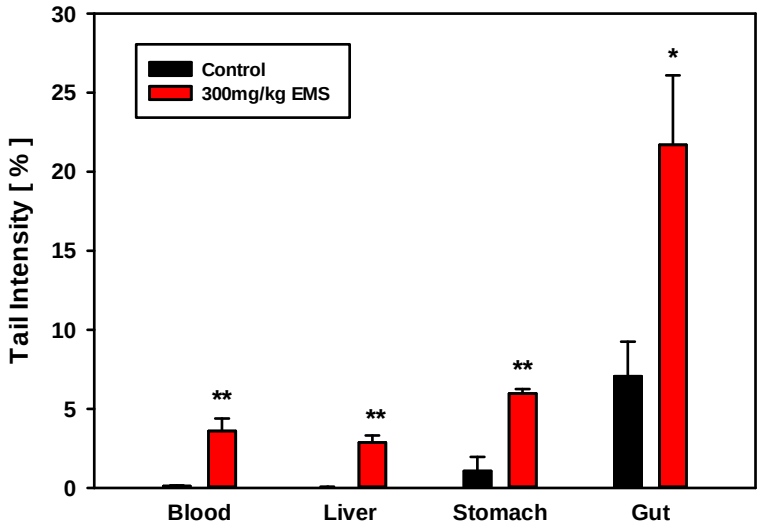


Figure 12 Results of the *in vivo* alkaline comet assay pre-study. Four hours before sacrifice 6 animals received orally 10 mL/kg body weight of water as vehicle (negative control) or 10 mL/kg body weight of an EMS solution (30 mg/mL). Data represent group means ± SE of the medians of 50 nuclei per slide and two slides per animal. * / ** significantly different from the respective negative controls: $P \leq 0.05$ / $P \leq 0.01$. Mann-Whitney U-Test.

19. Results of the combined mammalian erythrocyte micronucleus test and *in vivo* alkaline comet assay with Alternariol in mice

19.1. Observations and body weights

The test item AOH didn't induce treatment-related mortality or any signs of acute toxicity in the male NMRI mice, used for both the mammalian erythrocyte micronucleus test and the *in vivo* alkaline comet assay. By considering all treated animals, only in two of the AOH-treated animals slight piloerection was noted at the day of the last application/sacrifice. But concerning body weights, a reduction in body weight, instead of body weight gain, was observed for nine of 13 vehicle control animals (group mean body weight development: -0.54 ± 1.232 g within 48 h; range: -3.1 to 1.1 g) and eight of ten AOH-treated animals (group mean body weight development: -1.04 ± 1.100 g within 48 h; range: - 2.7 to + 0.9 g) between the first and last day of application. As the mean body weight development of the CP-treated animals (one oral application in tap water, 24 h before sacrifice and no overnight starving before weighting at the day of sacrifice) in the micronucleus test amounted to + 2.92 ± 1.910 g after 24 h with no animal demonstrating body weight loss, the three applications with corn oil ± AOH, with overnight starving prior to administration, obviously impeded body weight gain. For the AOH-treated animals reduction in body weight reached statistical significance ($P > 0.05$).

19.2. Histopathology

Pathological as well as histopathological investigations were done for all animals to exclude false positive results in the *in vivo* alkaline comet assay due to excess cytotoxicity of the test item AOH. There were no visible macroscopic findings in any of the NMRI mice, as examined during section. Using formalin-fixed tissue, very slight, (multi)focal inflammatory cell infiltration was noted within the liver of five vehicle controls animals, three AOH-treated animals and one EMS-treated animal. This lesion represents a common background finding in mice and was thus judged to be without relation to treatment. In the mucosa of the glandular stomach of one corn oil-treated animal a very slight focal epithelial degeneration was found. However, this area was so small in comparison to the remaining normal mucosa that influence on the respective comet assay results seems unlikely. Interestingly, within the jejunum of five animals and in the duodenum of one animal of the EMS-treated group very slight luminal cellular debris was visible within the crypts. But the amount of degenerative cells was very small in comparison to the remaining unchanged cells. Thus, no influence on the results of the comet assay can be expected. All other investigated tissues such as bone marrow, cecum, colon, oesophagus, forestomach, gall bladder, ileum and rectum were within normal limits. In conclusion, the histopathological investigation revealed neither necrosis or degenerative changes of the epithelium of the gastrointestinal tract nor changes within the liver that might have interfered with the conducted investigations.

19.3. Mammalian erythrocyte micronucleus test

The mammalian erythrocyte micronucleus test *in vivo* with bone marrow of male NMRI mice was performed to assess the potential of the food contaminant AOH to induce DNA/chromosome damage and/or to disturb chromosome distribution after repeated exposure (3 x 2000 mg/kg). The results of the mammalian erythrocyte micronucleus test will be described in more detail in the GLP-conform final report. This report will be available on request.

<u>Bone marrow cytotoxicity</u>

As assessed by differential cell counting of bone marrow smears, AOH in the given dose (3 x 2000 mg/kg body weight) did not significantly influence red blood cell (RBC) formation in male NMRI mice (see Table 22). The mean ratio of polychromatic erythrocyte (PCE) to the whole RBC fraction amounted to 111 ± 9.8 PCE/200 RBC compared to 107 ± 14.2 PCE/200 RBC for the vehicle control. The positive control CP also demonstrated no repression of RBC formation with a mean

number of 107 ± 9.6 PCE/200 RBC.

Table 22 Red blood cell formation and micronucleus induction in bone marrow cells of male NMRI mice after oral exposure to corn oil (3 x 10 ml/kg), AOH (3 x 2000 mg/kg) or cyclophosphamide monohydrate (1 x 60 mg/kg)

Treatment group	Dose sampling time	PCE/200 RBC	PCE:NCE	MN/2000 PCE	% MN PCE
Vehicle control. **Corn oil**	3 x 10 ml/kg b.w. p.o. 48 h	106.6 ± 14.24	1.18 ± 0.325	3.8 ± 3.56	0.19
Positive control. **CP**	60 mg /kg b.w. p.o. 24 h	107.4 ± 9.58	1.18 ± 0.232	79.0 ± 10.56**	3.95
Test item. **AOH**	3 x 2000 mg/kg b.w. p.o. 48 h	111.2 ± 9.76	1.28 ± 0.266	3.0 ± 1.41	0.15

PCE: Polychromatic erythrocytes; NCE: Normochromatic erythrocytes; RBC: Red blood cells; MN: Micronuclei; % MN PCE: Percent micronucleated PCE; Significantly different from vehicle controls: **. $P \leq 0.01$. U-test according to Mann-Whitney (two-group comparisons versus vehicle controls).

Micronucleus induction

Under the specific treatment conditions used, AOH did not significantly enhance the number of micronuclei in PCE of the bone marrow of male NMRI mice (see Table 22). Mean micronucleus frequency of the AOH-treated mice amounted to 3.0 ± 1.41 micronuclei/2000 PCE (range: 2 to 5), compared to 3.8 ± 3.56 micronuclei/2000 PCE (range: 1 to 10) for the vehicle control group. All vehicle- and AOH-treated animals, except for one animal of the vehicle control group (10 micronuclei/2000 PCE), exhibited micronucleus frequencies within the historical negative control range for male NMRI mice 48 h after treatment (historical range: 1 to 9 micronuclei/2000 PCE). In contrast, as expected, the positive control CP significantly induced micronucleus formation in PCE of the bone marrow, with a mean micronucleus frequency of 79.0 ± 10.56 micronuclei/2000 PCE (range: 67 to 88; $P \leq 0.01$). Micronucleus frequencies of all positive controls fell within the range of the historical positive controls 24 h after treatment (historical positive control range: 58 to 221).

Conclusions

As there was no bone marrow toxicity, the vehicle controls demonstrated low spontaneous micronucleus frequencies, and the positive controls exhibited significantly higher micronucleus frequencies, compared to the vehicle controls, the present mammalian erythrocyte micronucleus test was performed properly and can be judged as valid. Under the restrictions of this assay and the experimental conditions and dose used Alternariol (AOH) is considered non-mutagenic in immature bone marrow erythrocytes (PCE) of male NMRI mice.

19.4. *In vivo* alkaline comet assay

The *in vivo* alkaline comet assay with stomach, gut, and liver of male NMRI mice was performed to assess the potential of the food contaminant AOH to induce DNA-single and –double strand breaks after repeated exposure (3 x 2000 mg/kg) and thus direct genotoxicity in the organs of first contact, after oral application, as well as in one systemic organ. The results of the study will be presented in more detail in the GLP-conform final report depicting all results and relevant data of the combined mammalian erythrocyte micronucleus and *in vivo* alkaline comet assay study.

Tail intensity

For data evaluation of the *in vivo* alkaline comet assay group mean tail intensities, derived from four up to ten animals per group and organ and the mean of the medians of three slides per organ were used as the primary endpoints. Data for all groups and organs are given in Table 24. Three of 13 vehicle control animals had to be eliminated from statistical analyses due to a technical problem with the agarose temperature during slide generation, which let to artificially high TI values. For some animals in both treatment groups, slides generated from gut tissue were not analysable (see Figure 13 for representative slide regions of all treatment and organs). For this reason, the number of analysable animals concerning gut tissue and TI was reduced to 7 (AOH and EMS treatment) and 4 animals (vehicle control). Concerning analysis of hedgehogs, plausible analysis was impossible for all gut samples in the vehicle control group and for 5 animals in the other two treatment groups because these slides demonstrated > 95 % hedgehogs.

Liver: In the liver, as systemic organ, corn oil- and AOH-treated animals demonstrated comparable group mean TI of 0.9 ± 0.48 and 0.9 ± 1.42 %, respectively, indicating no systemic clastogenic effect of AOH in NMRI mice, which was in line with the results of the micronucleus test. When compared with the pre-study (mean TI: 0.1 ± 0.02 %), group mean TI of the vehicle controls and also the TI values of the single animals were higher, perhaps pointing to some effect of corn oil as vehicle. Maybe, for this reason the EMS positive control indeed demonstrated a higher group mean TI value of 1.3 ± 0.53, but the difference between vehicle and positive control did not reach statistical significance. EMS was applied only once in tap water.

Stomach: In the stomach, as the tissue of first contact, the vehicle control animals unexpectedly demonstrated the highest group mean TI of 25.9 ± 14.43 % with a very wide range of the means of the medians for the different animals. In contrast, the AOH-treated animals (lower “corn oil” dose than the vehicle control, due to high amount of AOH) showed a lower group mean TI value of 9.2 ± 7.83 % with a markedly lower range. The positive control EMS (applied in tap water) exhibited the lowest group mean TI value, amounting to 5.3 ± 2.39 with a markedly lower range compared to both corn oil and AOH. As the EMS group mean TI value resembled that of the pre-study (6.1 ± 0.29 %), the methodology seemed to be performed adequately, but there seemed to be disturbance of slide generation by corn oil treatment of the animals. This was also obvious by a different behaviour of the tissue when isolating the cells by scraping. This unwanted effect of corn oil was reproducible when analysing additional animals in a repeat test. For this reason, no conclusions can be drawn concerning direct genotoxic activity of AOH in the stomach.

Gut: Gut tissue seemed to be even more prone to the disturbing effect of corn oil treatment on generation of tissue slides for the *in vivo* alkaline comet assay. In the vehicle control group only 4 of 13 animals could be analysed. The only animal group which could be analysed adequately, was the positive control group (EMS, applied in tap water) with a group mean TI value of 20.1 ± 8.82%, which was nearly identical to the group mean TI value of the pre-study (21.5 ± 4.39 %), again indicating correct performance of the test. Unfortunately, also no conclusions can be drawn concerning direct genotoxic activity of AOH in the gut.

Table 23 Analysable animals, group mean tail intensities, and tail intensity ranges of liver, stomach, and gut tissue from male NMRI mice after oral exposure to corn oil (3 x 10 mL/kg), AOH (3 x 2000 mg/kg), or ethyl methanesulfonate (1 x 300 mg/kg)

Tissue	Treatment group	Treatment	Analysable animals	TI [%] group mean ± SD	TI [%] range
Liver	1	Corn oil	10	0.9 ± 0.48	0.2 – 1.4
	2	AOH	10	0.9 ± 1.42	0.1 – 4.4
	4	EMS	10	1.3 ± 0.53	0.6 – 2.1
Stomach	1	Corn oil	10	25.9 ± 14.43	7.3 – 46.1
	2	AOH	10	9.2 ± 7.83	3.4 – 28.7
	4	EMS	10	5.3 ± 2.39	3.0 – 10.8
Gut	1	Corn oil	4	45.9 ± 9.9	32.2 – 54.9
	2	AOH	7	43.0 ± 19.70	3.9 – 67.0
	4	EMS	7	20.1 ± 8.82	6.2 – 35.4

AOH: Alternariol; EMS: Ethyl methanesulfonate; TI: Tail intensity; SD: Standard deviation. Data were rounded to get one decimal place only.

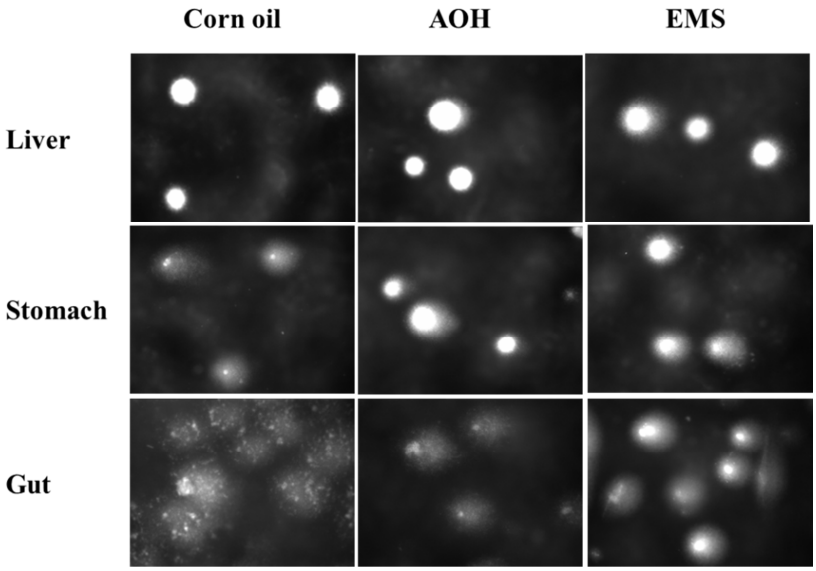


Figure 13 Representative pictures of slide regions from the *in vivo* alkaline comet assay with liver, stomach, and gut tissue from male NMRI mice after oral exposure to corn oil (3 x 10 ml/kg), AOH (3 x 2000 mg/kg), or ethyl methanesulfonate (1 x 300 mg/kg)

Hedgehogs

Analysis of percent hedgehogs was used, besides histopathology, to get an idea concerning cytotoxic effects of AOH in the analysed tissues (see Table 24). In the liver no hedgehogs (vehicle control) or only a very small percent of hedgehogs (AOH and EMS treatment) was noted, indicating accurate performance of the test and no AOH-related hepatotoxic activity. For stomach and gut percent hedgehogs well reflected the situation, as also observed by TI analysis. For the corn oil slides no meaningful data could be generated for gut tissue. In addition, for the stomach percent hedgehogs were also maximal for the vehicle control animals, with a lower percentage of hedgehogs for the slides of AOH-treated and EMS-treated animals.

Table 24 Analysable animals, percent hedgehogs, and ranges for hedgehogs of liver, stomach, and gut tissue from male NMRI mice after oral exposure to corn oil (3 x 10 ml/kg), AOH (3 x 2000 mg/kg), or ethyl methanesulfonate (1 x 300 mg/kg)

Tissue	Treatment group	Treatment	Analysable animals	Hedgehogs [%]	Hedgehogs range: sum/3 slides
Liver	**1**	**Corn oil**	10	0.0	0 – 0
	2	**AOH**	10	0.2	0 – 2
	4	**EMS**	10	0.7	0 – 8
Stomach	**1**	**Corn oil**	10	7.1	1 – 26
	2	**AOH**	10	2.5	0 – 9
	4	**EMS**	10	2.3	0 – 11
Gut	**1**	**Corn oil**	n.a.	n.a.	n.a.
	2	**AOH**	5	5.1	5 – 28
	4	**EMS**	5	5.7	9 - 28

AOH: Alternariol; EMS: Ethyl methanesulfonate; n.a.: Not analysable, > 95 % hedgehogs.

Conclusions

As the EMS-treated animals demonstrated group mean TI values which clearly resembled that of the pre-study, and especially for liver tissue, nearly no significant induction of DNA-strand breaks and occurrence of hedgehogs was noted, the methodology per se was performed properly. Based on the received data it can be concluded, that under the restrictions of this assay and the experimental conditions and dose used, Alternariol (AOH) does not induce direct systemic genotoxicity in liver tissue of male NMRI mice. This is in line with the absence of micronucleus induction in bone marrow cells in the parallel mammalian erythrocyte micronucleus test. Unfortunately, no judgement can be made on local genotoxicity of AOH in the gastrointestinal tract, as in the absence of significant treatment-related histopathologic changes, corn oil treatment, perhaps in combination with overnight starving, seems to interfere with tissue/slide preparation from stomach and gut, perhaps by decreasing cells' resistance to mechanical and/or chemical stress. Consequently, investigation of local genotoxicity of AOH in the gastrointestinal tract by the *in vivo* alkaline comet assay seems only possible by using other application modes than oral gavage in corn oil, for example feeding of AOH.

CONCLUSIONS AND RECOMMENDATIONS

The results of the present study suggest that only marginal amounts accounting for less than 0.1% of the dose applied become bioavailable and reach the systemic circulation after oral application. The main proportion of the oral dose (85 to 91 %) is rapidly excreted vie faeces. Up to 11 % of the total radioactivity dose was recovered in urine. This result is inconsistent with the low radioactivity levels detected in blood and tissues, as it has to generally be assumed that the fraction that is excreted via urine has been absorbed before. We thus conclude that AOH undergoes extensive first pass metabolism in the liver and that the fraction absorbed from the intestine is in large part rapidly metabolized and excreted via urine without reaching the systemic circulation. This hypothesis is supported by the fact that only 0.1 to 6 % of the dose applied was detected in urine as AOH or hydroxylated metabolites in the non-radioactive study. This result suggests that other as yet unidentified metabolites contribute to the radioactivity recovered in the urinary fraction. Most likely, AOH undergoes conjugation reactions to form glucoronides or sulphates. For future studies, we recommend non-target analysis of AOH metabolites in blood and urine in order to gain insight into the metabolic fate of AOH.

Mice tolerated the oral application of AOH up to doses of 2000 mg/kg BW without any impairment of their general health status. Furthermore, AOH did not significantly enhance the number of micronuclei in PCE of the bone marrow of male NMRI mice and is thus considered non-mutagenic in immature bone marrow erythrocytes (PCE) of male NMRI mice. In addition, the *in vivo* alkaline comet assay with liver tissue of AOH-exposed NMRI mice also indicated lack of systemic genotoxicity of AOH. Lack of a significant toxic/genotoxic potential of AOH *in vivo* might thereby be based on the limited amounts of AOH reaching systemic circulation, on rapid metabolism to non-(geno)toxic metabolites excreted via urine, or also real absence of marked genotoxicity in systemic tissues. As assessed in blood of five animals, in parallel to the combined mammalian erythrocyte micronucleus test and *in vivo* alkaline comet assay, blood concentrations of about 0.5 µM AOH were reached three hours after the last application of 3 x 2000 mg/kg body weight. This concentration range was also used in the *in vitro* assays with AOH and gave mostly borderline or negative results. Interestingly, *in vitro* mutagenicity tests with AOH were always performed without an external metabolizing system, like S9-mix, thus ignoring potential metabolic inactivation of AHO, which is likely to occur *in vivo*, and which might be responsible for the lack in systemic genotoxicity. For examples Fleck et al. (4) investigated mutagenicity of AOH and other *Alternaria* toxins *in vitro*, using the *HPRT* mutagenicity test with V79 cells, a cell type with a short doubling time and nearly no potential to metabolize foreign substances. They detected a significant increase in mutant frequency from 10 µM upwards. By considering the absence of systemic genotoxicity and marked cytotoxicity in the present study, irrespective of a very high dose, which was supported by the parallel histopathological investigation of a broad panel of organs, the obviously high metabolic modification of AOH *in vivo* and also the lack of data concerning genotoxicity in the presence of a metabolizing system, AOH seems relatively safe when reaching systemic circulation. For a judgement concerning local genotoxicity in the gastrointestinal tract however, it would need further investigations with another application mode than suspending AOH in corn oil. Interestingly, an *in vitro* study by Fleck et al (4), who investigated the genotoxicity of several *Alternaria* mycotoxins using the *HPRT* test and the alkaline DNA unwinding assay, pointed to a significantly about 50-times higher mutagenicity of another *Alternaria* toxin, i.e. Altertoxin II, compared to AOH and alternariol methyl ether. Furthermore, Schwarz et al. (5), tested extracts of *Alternaria alternata* infested rice, which were fractionated using preparative HPLC. The resulting fractions were subsequently tested for genotoxicity using the FPG-modified comet assay and the fractions compared to single compounds. From their results, the authors concluded that the main secondary metabolites of AOH, i.e. tenuazonic acid and alternariol monomethyl ether, play only a minor role for the genotoxic properties of the extracts. Thus, AOH seems not to play the predominant role for toxicity and genotoxicity in the group of *Alternaria* toxins. Studies aiming to identify the highly mutagenic compounds produced by *Alternaria* species are warranted.

The biggest obstacle in performing a toxicokinetic study with mycotoxins is the sourcing of sufficient amounts/quantities of the test materials and the respective internal standards for subsequent quantitative bioanalysis. A feasible approach to overcome this issue might be the extraction of mycotoxins from mould cultures rather than attempting their costly synthesis. The replacement of D-glucose by ^{13}C labelled D-glucose in the culture media can be used to facilitate the biosynthesis of ^{13}C labelled mycotoxins, which can then be applied as internal standards in LC-MS based bioanalysis / validation. The unambiguous identification of the mycotoxins obtained this way can be achieved by NMR spectroscopy in combination with LC-MS.

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[3] Pfeiffer E, Schebb NH, Podlech J and Metzler M, 2007. Novel oxidative metabolites of the mycotoxins alternariol and alternariol methyl ether. Molecular Nutrition and Food Research, 51, 307-316. 6

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[5] Schwarz S, Kreutzer M and Marko D, 2012.Minor contribution of alternariol, alternariol monomethyl ether and tenuazonic acid to the genotoxic properties of extracts from *Alternaria Alternata* infested rice. Toxicology Letters, 214, 46-52.

APPENDICES

Appendix A. 1H NMR spectra of the oxidized metabolites

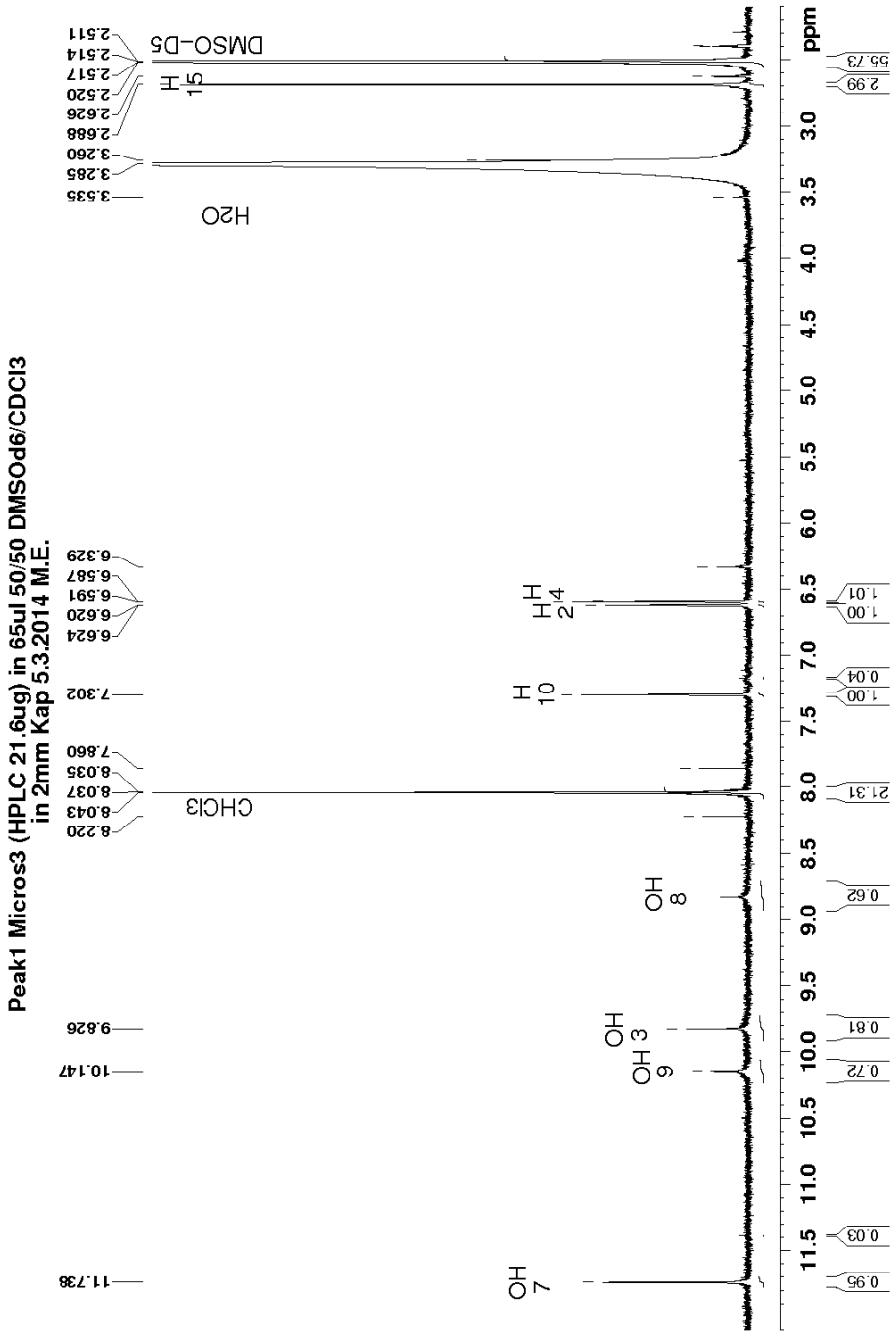

Figure 14 Annotated ^{1}H NMR spectrum of 8-hydroxy-AOH (peak 1) dissolved in $CDCl_3$/DMSO-D_6 (50:50)

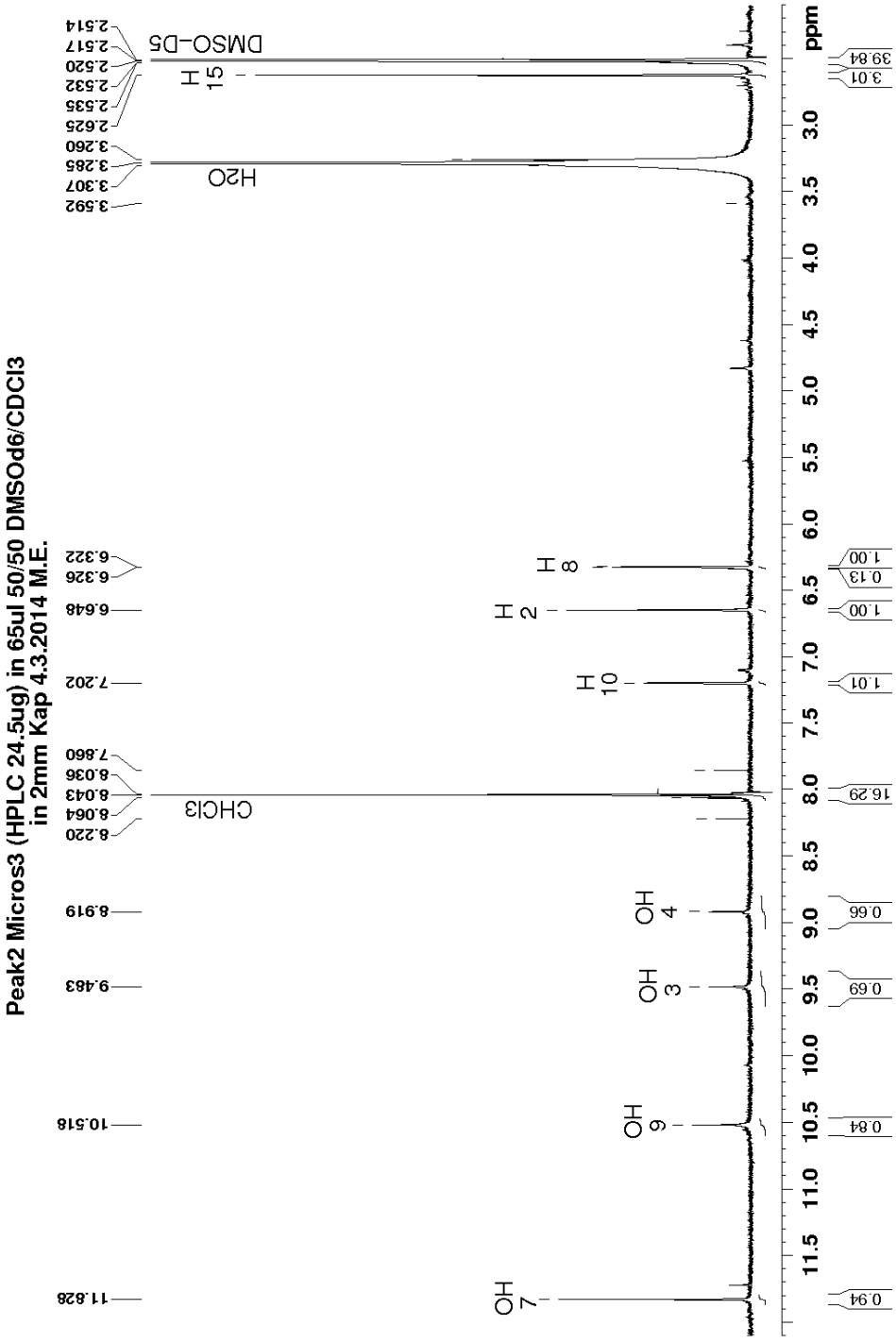

Figure 15 Annotated ^{1}H NMR spectrum of 4-hydroxy-AOH (peak 2) dissolved in $CDCl_3$/DMSO-D_6 (50:50)

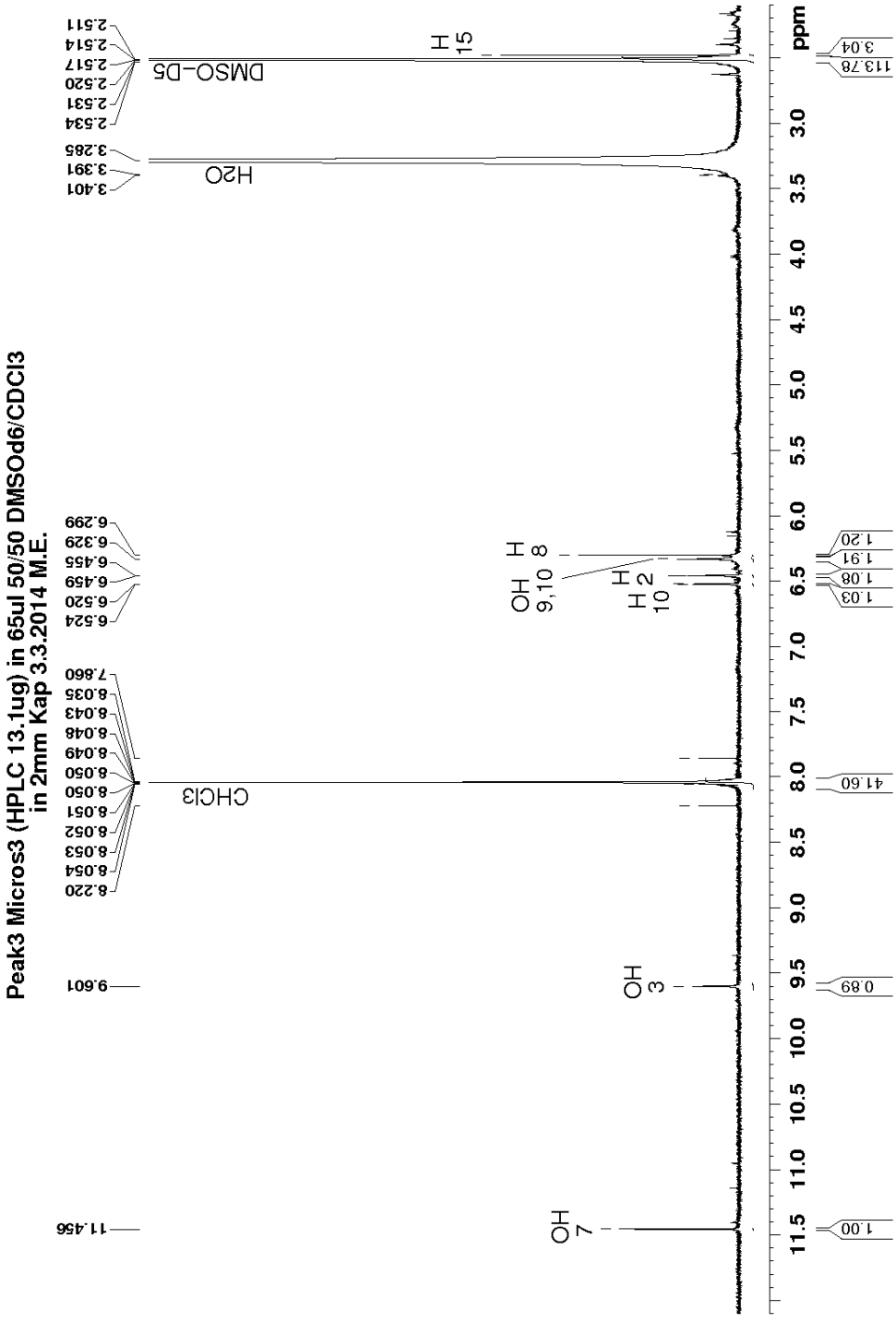

Figure 16 Annotated ^{1}H NMR spectrum of 10-hydroxy-AOH (peak 3) dissolved in $CDCl_3$/DMSO-D_6 (50:50)

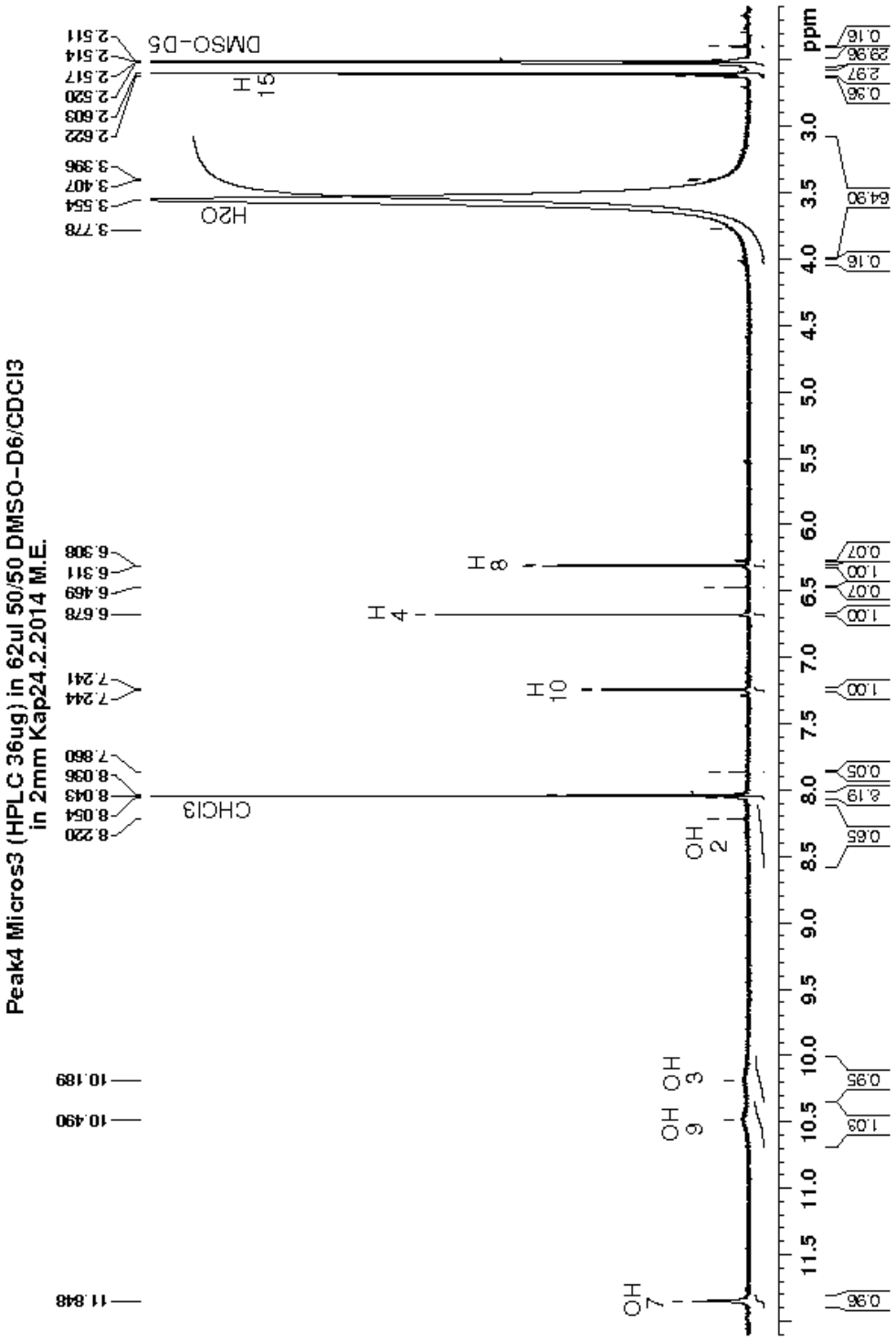

Figure 17 Annotated ^{1}H NMR spectrum of 2-hydroxy-AOH (peak 4) dissolved in $CDCl_3$/DMSO-D_6 (50:50)

Appendix B. MS spectra of the oxidized metabolites

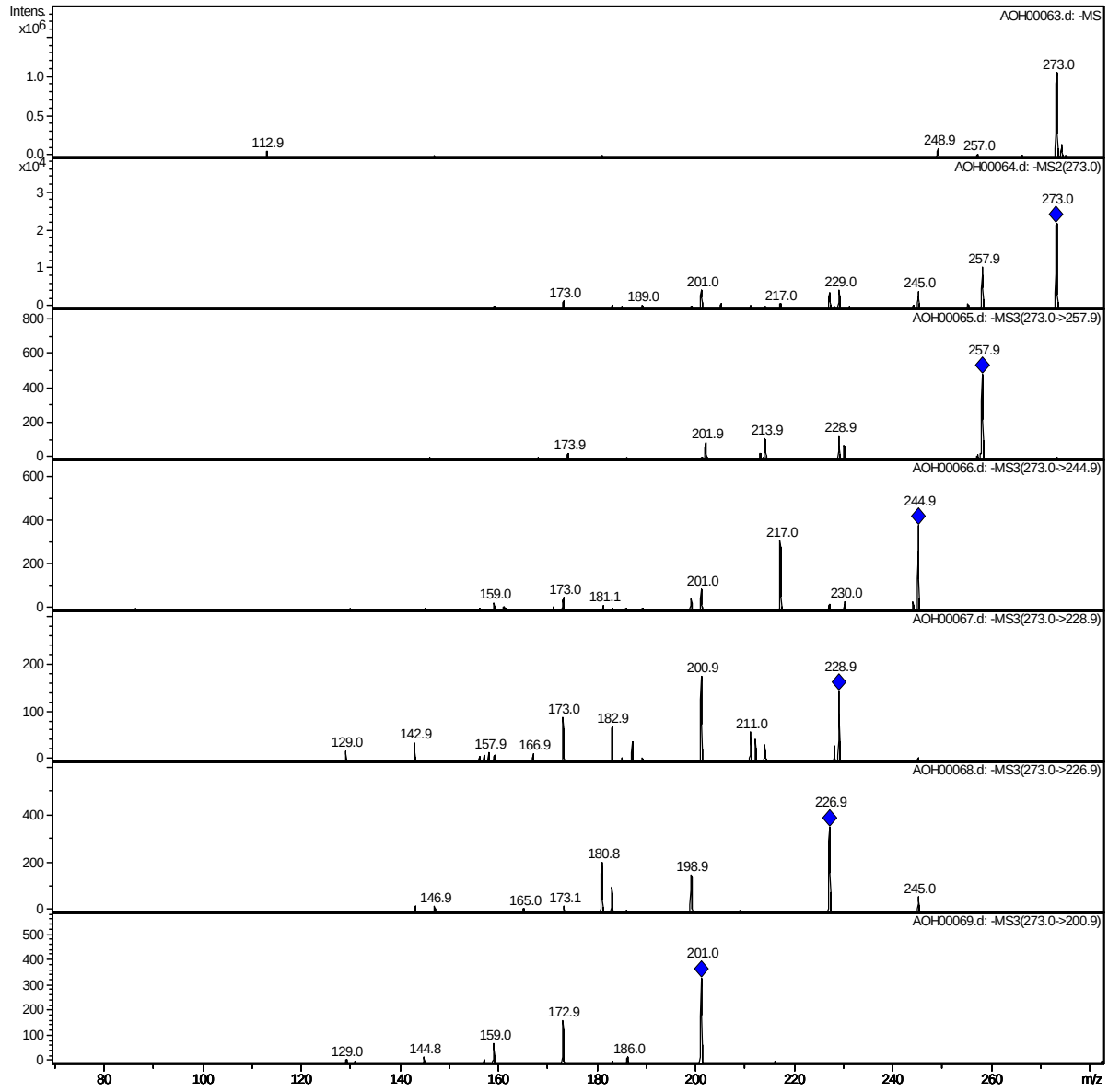

Figure 18 MS (uppermost) and MS fragmentation spectra of 8-Hydroxy-AOH (Peak 1) in the negative ion mode. The blue diamond always indicates the selected pre-cursor ion in the fragmentation spectra. The main fragments obtained in MS^2 (second spectrum from the top) were further fragmented in the subsequent MS^3 spectra.

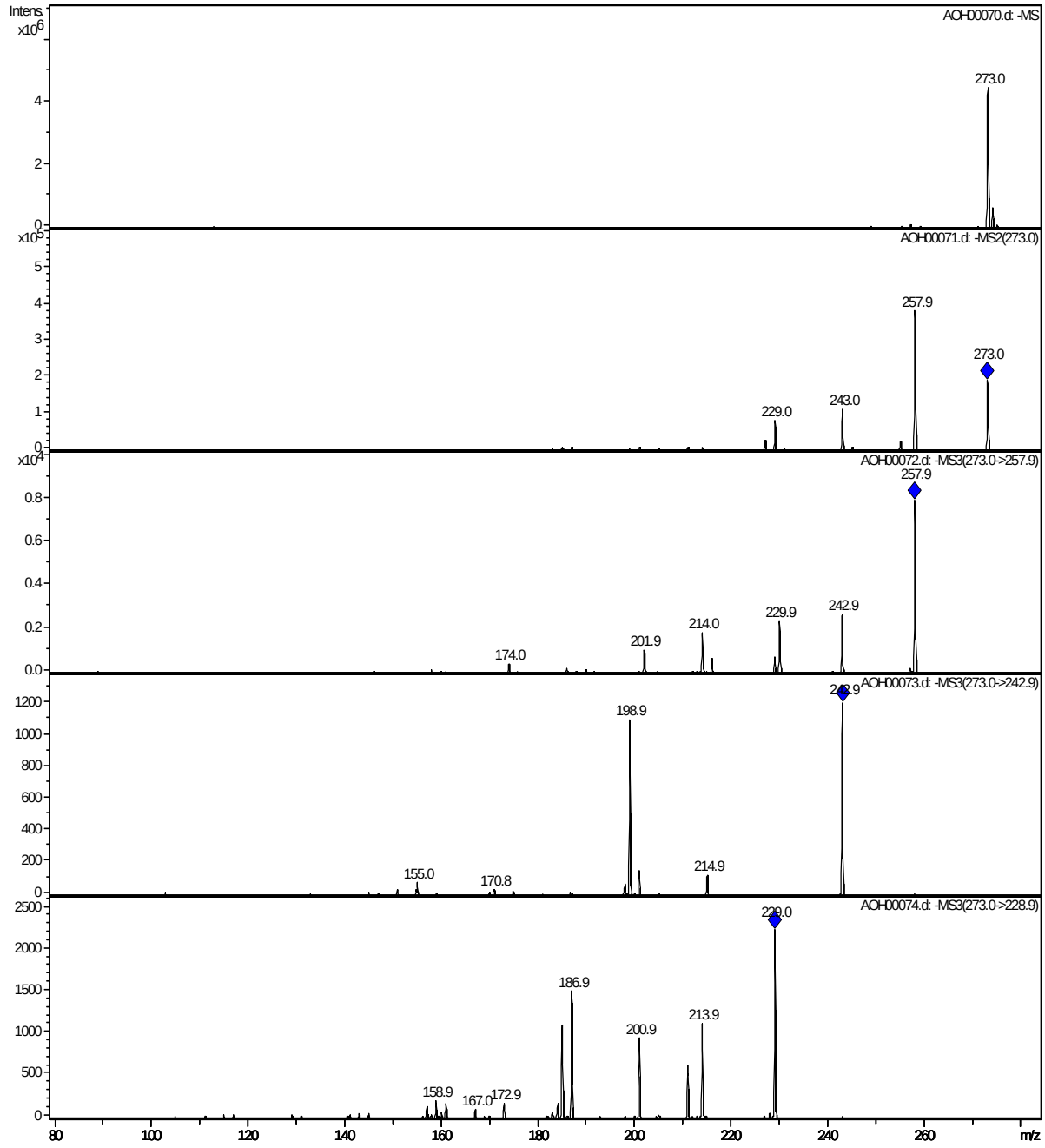

Figure 19 MS (uppermost) and MS fragmentation spectra of 4-Hydroxy-AOH (Peak 2) in the negative ion mode. The blue diamond always indicates the selected pre-cursor ion in the fragmentation spectra. The main fragments obtained in MS^2 (second spectrum from the top) were further fragmented in the subsequent MS^3 spectra.

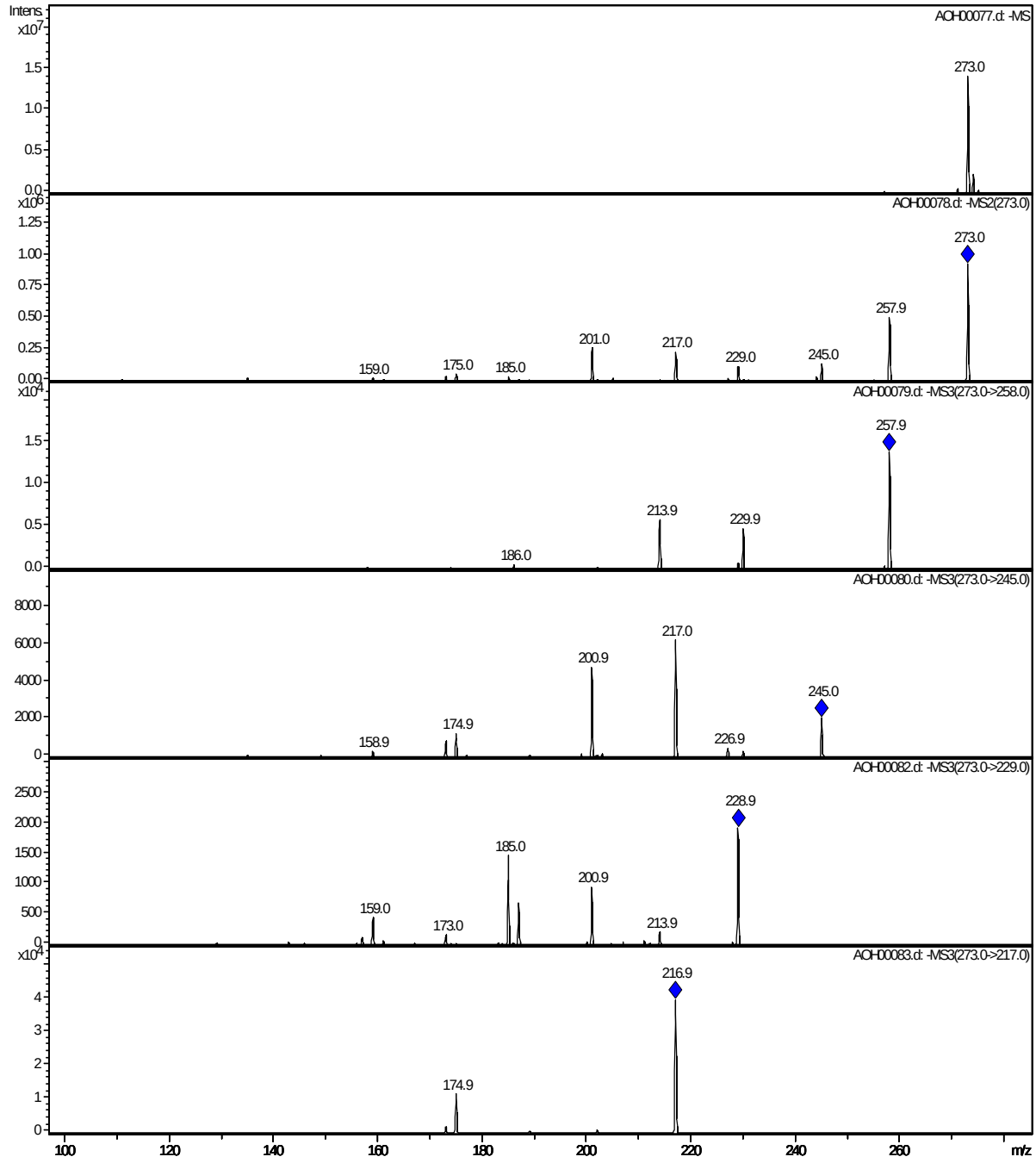

Figure 20 MS (uppermost) and MS fragmentation spectra of 10-Hydroxy-AOH (Peak 3) in the negative ion mode. The blue diamond always indicates the selected pre-cursor ion in the fragmentation spectra. The main fragments obtained in MS^2 (second spectrum from the top) were further fragmented in the subsequent MS^3 spectra.

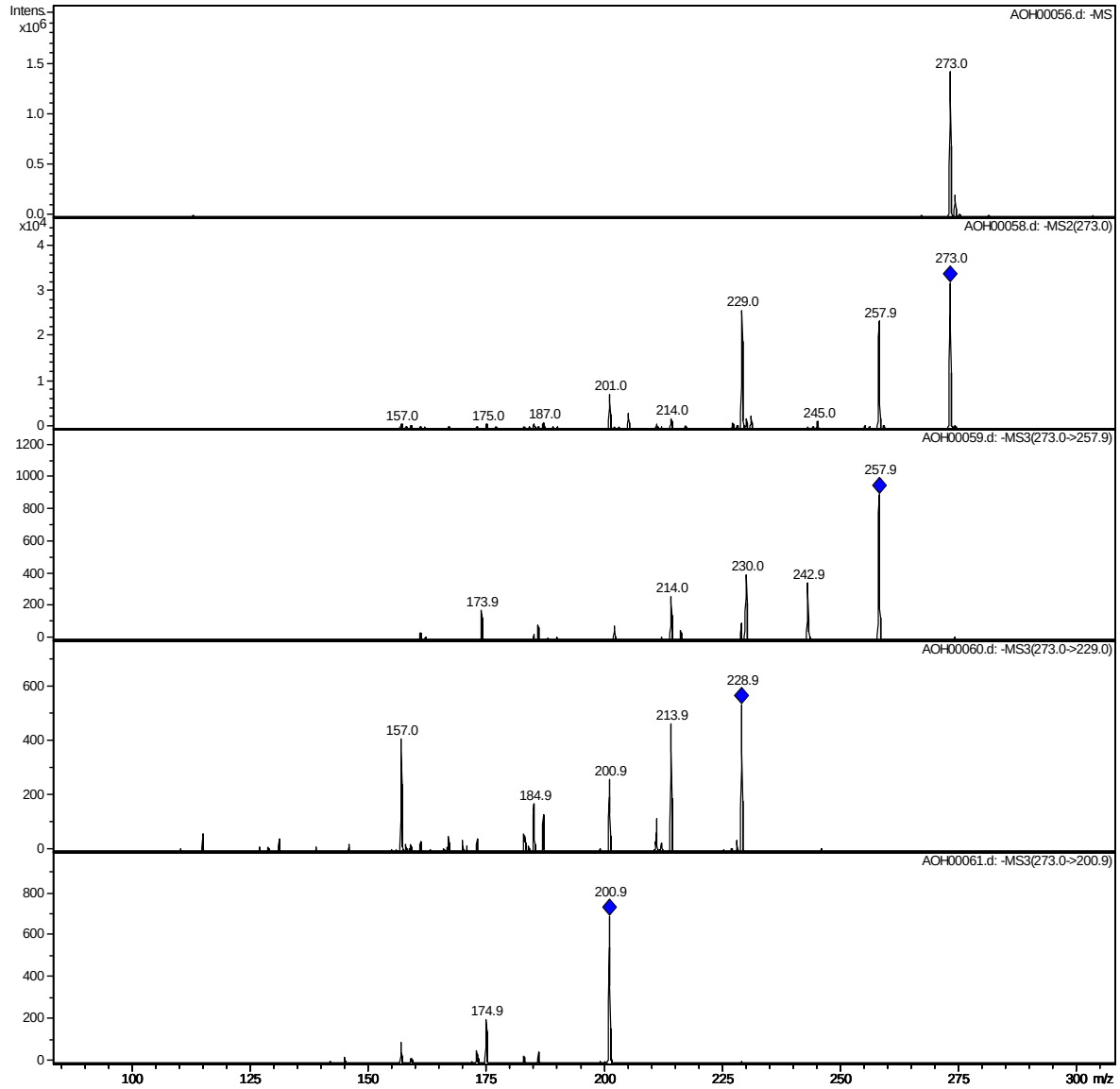

Figure 21 MS (uppermost) and MS fragmentation spectra of 2-Hydroxy-AOH (Peak 4) in the negative ion mode. The blue diamond always indicates the selected pre-cursor ion in the fragmentation spectra. The main fragments obtained in MS^2 (second spectrum from the top) were further fragmented in the subsequent MS^3 spectra.

Appendix C. 2D NMR spectra of the oxidized metabolites

Table 25 Observed COSY crosses of 8-Hy-droxy-AOH (Peak 1) measured in $CDCl_3$/ DMSO-D_6 (50:50)

	H-2	H-4	H-10	H-15	OH-7
H-2		X		X	
H-4	X			X	
H-10				X	
H-15	X	X	X		
OH7					

Table 26 Cross peaks observed in HSQC (black) and HMBC (red) NMR spectra of 8-Hydroxy-AOH (Peak 1) measured in $CDCl_3$/DMSO-D_6 (50:50). Number of coupling bonds and type of coupling are given according to the current nomenclature.

	H-2	H-4	H-10	H-15	OH-3	OH-7	OH-8	OH-9
C-1				$2J_{CH}$				
C-2	$1J_{CH}$	$3J_{CH}$		$3J_{CH}$				
C-3	$2J_{CH}$							
C-4	$3J_{CH}$	$1J_{CH}$						
C-6								
C-7						$2J_{CH}$		
C-8			$3J_{CH}$			$3J_{CH}$		
C-9			$2J_{CH}$					
C-10			$1J_{CH}$	5J				
C-11								
C-12			$3J_{CH}$			$3J_{CH}$		
C-13	$3J_{CH}$	$3J_{CH}$	$3J_{CH}$	$3J_{CH}$				
C-14		2J						
C-15				$1J_{CH}$				

only found in spectra with sharp OH-signals

Table 27 Observed ROESY crosses of 8-Hydroxy-AOH (Peak 1) measured in $CDCl_3$/DMSO-D_6 (50:50)

	H-2	H-4	H-10	H-15	OH-3	OH-7	OH-8	OH-9
H-2				m	w			
H-4					w			
H-10				S				w
H-15	m		S					
OH-3	w	w						
OH-7								
OH-8								
OH-9			w					

S = strong. m = medium. w = weak

Table 28 Observed COSY crosses of 4-Hy-droxy-AOH (Peak 2) measured in $CDCl_3$/ DMSO-D_6 (50:50)

	H-2	H-8	H-10	H-15	OH-7
H-2				**X**	
H-8			**X**		
H-10		**X**		**X**	
H-15	**X**		**X**		
OH7					

Table 29 Cross peaks observed in HSQC (black) and HMBC (red) NMR spectra of 4-Hydroxy-AOH (Peak 2) measured in $CDCl_3$/DMSO-D_6 (50:50). Number of coupling bonds and type of coupling are given according to the current nomenclature.

	H-2	H-8	H-10	H-15	OH-3	OH-4	OH-7	OH-9
C-1				**$2J_{CH}$**				
C-2	**$1J_{CH}$**			**$3J_{CH}$**				
C-3	**$2J_{CH}$**							
C-4	**$3J_{CH}$**				**$3J_{CH}$**			
C-6								
C-7		**$2J_{CH}$**					**$2J_{CH}$**	
C-8		**$1J_{CH}$**	**$3J_{CH}$**				**$3J_{CH}$**	
C-9			**$2J_{CH}$**					**$2J_{CH}$**
C-10		**$3J_{CH}$**	**$1J_{CH}$**	**$5J_{CH}$**				**$3J_{CH}$**
C-11								
C-12		**$3J_{CH}$**	**$3J_{CH}$**				**$3J_{CH}$**	
C-13	**$3J_{CH}$**		**$3J_{CH}$**	**$3J_{CH}$**				
C-14								
C-15				**$1J_{CH}$**				

Table 30 Observed ROESY crosses of 4-Hydroxy-AOH (Peak 2) measured in $CDCl_3$/DMSO-D_6 (50:50)

	H-2	H-8	H-10	H-15	OH-3	OH-4	OH-7	OH-9
H-2				**m**	**w**			
H-8							**w**	**w**
H-10				**S**				**w**
H-15	**m**		**S**					
OH-3	**w**							
OH-4								
OH-7		**w**						
OH-9		**w**	**w**					

S = strong. m = medium. w = weak

Table 31 Observed COSY crosses of 10-Hy-droxy-AOH (Peak 3) measured in $CDCl_3$/ DMSO-D_6 (50:50)

	H-2	H-4	H-8	H-15	OH-7
H-2		**X**		**X**	
H-4	**X**			**X**	
H-8					
H-15	**X**	**X**			
OH7					

Table 32 Cross peaks observed in HSQC (black) and HMBC (red) NMR spectra of 10-Hydroxy-AOH (Peak 3) measured in $CDCl_3$/DMSO-D_6 (50:50). Number of coupling bonds and type of coupling are given according to the current nomenclature.

	H-2	H-4	H-8	H-15	OH-3	OH-7
C-1				**$2J_{CH}$**		
C-2	**$1J_{CH}$**			**$3J_{CH}$**		
C-3						
C-4		**$1J_{CH}$**				
C-6						
C-7			**$2J_{CH}$**			**$2J_{CH}$**
C-8			**$1J_{CH}$**			**$3J_{CH}$**
C-9						
C-10			**$3J_{CH}$**			
C-11						
C-12			**$3J_{CH}$**			**$3J_{CH}$**
C-13	**$3J_{CH}$**			**$3J_{CH}$**		
C-14						
C-15				**$1J_{CH}$**		

Table 33 Observed ROESY crosses of 10-Hydroxy-AOH (Peak 3) measured in $CDCl_3$/DMSO-D_6 (50:50)

	H-2	H-4	H-8	H-15	OH-3	OH-7
H-2				**m**	**m**	
H-4					**m**	
H-8						
H-15	**m**					
OH-3	**m**	**m**				
OH-7						

S = strong. m = medium. w = weak

Table 34 Observed COSY crosses of 2-Hy-droxy-AOH (Peak 4) measured in $CDCl_3$/ DMSO-D_6 (50:50)

	H-4	H-8	H-10	H-15	OH-7
H-4				X	
H-8			X		
H-10		X		X	
H-15	X		X		
OH7					

Table 35 Cross peaks observed in HSQC (black) and HMBC (red) NMR spectra of 2-Hydroxy-AOH (Peak 4) measured in $CDCl_3$/DMSO-D_6 (50:50). Number of coupling bonds and type of coupling are given according to the current nomenclature.

	H-4	H-8	H-10	H-15	OH-2	OH-3	OH-7	OH-9
C-1				$2J_{CH}$				
C-2	$3J_{CH}$			$3J_{CH}$		$3J_{CH}$		
C-3	$2J_{CH}$					$2J_{CH}$		
C-4	**1JCH**					$3J_{CH}$		
C-6								
C-7		$2J_{CH}$					$2J_{CH}$	
C-8		$1J_{CH}$	$3J_{CH}$				$3J_{CH}$	$3J_{CH}$
C-9		$2J_{CH}$	$2J_{CH}$				$4J_{CH}$	$2J_{CH}$
C-10		$3J_{CH}$	$1J_{CH}$	$5J_{CH}$				$3J_{CH}$
C-11								
C-12		$3J_{CH}$	$3J_{CH}$				$3J_{CH}$	
C-13	$3J_{CH}$		$3J_{CH}$	$3J_{CH}$				
C-14	$2J_{CH}$							
C-15				$1J_{CH}$				

only found in spectra with sharp OH-signals

Table 36 Observed ROESY crosses of 2-Hydroxy-AOH (Peak 4) measured in $CDCl_3$/DMSO-D_6 (50:50)

	H-4	H-8	H-10	H-15	OH-2	OH-3	OH-7	OH-9
H-4						**m**		
H-8							**m**	**m**
H-10				**S**				**m**
H-15			**S**		**m**			
OH-2				**m**				
OH-3	**m**							
OH-7		**m**						
OH-9		**m**	**m**					

S = strong. m = medium. w = weak

Appendix D Results of AOH blood levels in male and female NMRI mice

Table 37 Mouse (male) Blood kinetics of AOH - Dose group 200mg/kg – blood (HPLC-MS)

Mouse – Male							
AOH [ng /mL]							
t [h]	A	B	C	D	Mean	STDEV	Rel STDEV [%]
predose	BLQ	–	–	–	BLQ*	NA	NA
0.5	49.9	44.3	64.9	53.0	53.0	7.53	14.2
1	22.8	47.6	42.7	48.2	40.3	10.3	25.6
2	29.0	37.0	88.6	110	66.2	34.1	51.6
3	16.6	24.7	17.0	34.0	23.1	7.09	30.7
4	13.7	BLQ	BLQ	BLQ	13.7*	NA	NA
5	BLQ	BLQ	BLQ	14.7	14.7*	NA	NA
6	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA
24	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA
48	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA

BLQ = Below limit of quantification (below 10.8 ng/mL)
NA = Not applicable
* = Single Value

Mouse – Male				
t [h]	A	B	C	D
predose	Anzeiger 1	–	–	–
0,5	2101	2102	2103	2104
1	2105	2106	2107	2108
2	2109	2110	2111	2112
3	2101	2102	2103	2104
4	2105	2106	2107	2108
5	2109	2110	2111	2112
6	2101	2102	2103	2104
24	2105	2106	2107	2108
48	2109	2110	2111	2112

Subject number code

1st Number	group
2nd Number	gender (1=male; 2=female)
3rd and 4th Number	animal ID

Table 38 Mouse (female) Blood kinetics of AOH - Dose group 200mg/kg – blood (HPLC-MS)

Mouse – Female							
AOH [ng /mL]							
t [h]	A	B	C	D	Mean	STDEV	Rel STDEV [%]
predose	BLQ	–	–	–	BLQ*	NA	NA
0.5	95.0	184	35.4	46.5	90.2	58.6	64.9
1	86.6	32.6	30.2	22.7	43.0	25.4	59.1
2	19.9	65.7	28.3	19.1	33.3	19.1	57.4
3	20.7	**291**#	33.5	51.8	35.3	12.8	36.1
4	BLQ	41.3	BLQ	BLQ	41.3*	NA	NA
5	21.2	34.5	43.6	14.7	28.5	11.3	39.5
6	BLQ	35.3	BLQ	18.8	27.1	NA	NA
24	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA
48	BLQ	BLQ	BLQ	BLQ	BLQ	NA	NA

BLQ = Below limit of quantification (below 10.8 ng/mL)
NA = Not applicable
* = Single Value

Mouse – Female				
t [h]	A	B	C	D
predose	Anzeiger 2	–	–	–
0,5	2201	2202	2203	2204
1	2205	2206	2207	2208
2	2209	2210	2211	2212
3	2201	2202	2203	2204
4	2205	2206	2207	2208
5	2209	2210	2211	2212
6	2201	2202	2203	2204
24	2205	2206	2207	2208
48	2209	2210	2211	2212

Subject number code

1st Number	group
2nd Number	gender (1=male; 2=female)
3rd and 4th Number	animal ID

Table 39 Calibration data AOH mice blood (HPLC-MS)

		AOH									Regression parameters		
		Nominal concentration [ng / mL]									y=a*x+b; 1/x weighting		
Date	**Matrix**	**10.8**	**26.9**	**53.8**	**86.0**	**108**	**269**	**430**	**538**	**774**	**Intercept**	**Slope**	**R²**
ID: 1													
12. Aug 14	mice blood	12.9	23.2	52.1	78.3	111	266	450	542	761			
Accuracy [%]		*120*	*86.3*	*96.9*	*91.0*	*103*	*99.0*	*105*	*101*	*98.3*	*0.0289*	*0.00441*	*0.9992*
ID: 2													
14. Aug 14	mice blood	12.8	25.2	53.2	79.9	99.9	266	441	681	780			
Accuracy [%]		*119*	*93.8*	*99.0*	*92.9*	*92.9*	*99.0*	*103*	*127**	*101*	*0.00295*	*0.00461*	*0.999*
ID: 3													
18. Aug 14	mice blood	12.3	25.8	48.5	79.2	111	263	454	568	733			
Accuracy [%]		*114*	*96.0*	*90.2*	*92.1*	*103*	*97.9*	*106*	*106*	*94.7*	*0.00996*	*0.00438*	*0.998*

* *Accuracy > 115 %; does not comply with the EMA-guideline acceptance criteria; standard was not included in regression*

Appendix E Results of urinary excretion of AOH and its metabolites in male and female mice

Table 40 Mouse (male) Dose group - 200 mg/kg AOH; oral application, urine (GC-MS) Urinary excretion of AOH and its metabolites

Mouse – male; Matrix: Urine								
AOH [ng] total								
t [h]	1101	1102	1103	1104	1105	Mean	STDEV	Rel STDEV [%]
24	5193	109	679	18861	652	5099	7122	140
48	12563	1578	14499	3169	1088	6579	5750	87.4
72	5260	1425	1220	1945	450	2060	1671	81.1

area -% peak area ratios of metabolites to IS were summed over all four metabolites and set in sum to 100%. The peak area ratios of the individual metabolites are accordingly indicated in% values.

Mouse – male; Matrix: Urine								
O3 = 10-hydroxy-AOH [area – %]								
t [h]	1101	1102	1103	1104	1105	Mean	STDEV	Rel STDEV [%]
24	5.01	5.77	NA	15.60	8.61	8.75	4.18	47.7
48	1.08	5.35	6.15	13.80	NA	6.59	4.58	69.5
72	14.3	20.3	48.2	20.9	14.1	23.5	12.6	53.7

Mouse – male; Matrix: Urine								
O2 = 4-hydroxy-AOH [area – %]								
t [h]	1101	1102	1103	1104	1105	Mean	STDEV	Rel STDEV [%]
24	42.1	52.0	NA	30.2	49.3	43.4	8.4	19.4
48	49.8	32.1	49.0	44.1	52.8	45.6	7.29	16.0
72	43.7	46.9	23.4	33.9	42.8	38.1	8.5	22.4

Mouse – male; Matrix: Urine								
O1 = 8-hydroxy-AOH [area – %]								
t [h]	1101	1102	1103	1104	1105	Mean	STDEV	Rel STDEV [%]
24	3.61	NA	NA	31.30	NA	17.46	NA	NA
48	6.95	NA	NA	NA	NA	6.95	NA	NA
72	3.14	5.65	NA	12.6	12.8	8.55	4.25	49.7

Mouse – male; Matrix: Urine								
O4 = 2-hydroxy-AOH [area – %]								
t [h]	1101	1102	1103	1104	1105	Mean	STDEV	Rel STDEV [%]
24	49.3	42.2	NA	22.90	42.1	39.1	9.8	25.1
48	42.1	62.6	44.8	42.1	47.2	47.8	7.63	16.0
72	38.9	27.2	28.4	32.7	30.3	31.5	4.15	13.2

Table 41 Mouse (female) Dosis group - 200 mg / kg AOH; oral application, urine (GC-MS) Urinary excretion of AOH and its metabolites

Mouse – female; Matrix: Urine								
AOH [ng] total								
t [h]	1201	1202	1203	1204	1205	Mean	STDEV	Rel STDEV [%]
24	23433	5172	240	5752	17595	10438	8646	83
48	596	1142	2140	2675	2469	1804	801	44.4
72	80	155	174	BLQ	BLQ	136	41	29.9

area -% peak area ratios of metabolites to IS were summed over all four metabolites and set in sum to 100%. The peak area ratios of the individual metabolites are accordingly indicated in% values.

Mouse – female; Matrix: Urine								
O3 = 10-hydroxy-AOH [area – %]								
t [h]	1201	1202	1203	1204	1205	Mean	STDEV	Rel STDEV [%]
24	8.62	24.4	69.6	15.7	27.6	29.2	21.3	72.9
48	27.6	21.0	4.96	16.6	22.3	18.5	7.61	41.2
72	64.9	47.5	40.6	41.0	58.7	50.5	9.74	19.3

Mouse – female; Matrix: Urine								
O2 = 4-hydroxy-AOH [area – %]								
t [h]	1201	1202	1203	1204	1205	Mean	STDEV	Rel STDEV [%]
24	52.1	38.1	10.2	50.8	34.9	37.2	15.1	40.6
48	25.1	30.5	47.4	18.9	37.2	31.8	9.83	30.9
72	3.18	7.87	18.2	NA	NA	9.76	6.29	64.4

Mouse – female; Matrix: Urine								
O1 = 8-hydroxy-AOH [area – %]								
t [h]	1201	1202	1203	1204	1205	Mean	STDEV	Rel STDEV [%]
24	NA	2.26	NA	NA	17.0	9.61	NA	NA
48	NA	2.35	2.43	NA	5.42	3.40	1.43	42.1
72	NA	NA	NA	NA	NA	NA	NA	NA

Mouse – female; Matrix: Urine								
O4 = 2-hydroxy-AOH [area – %]								
t [h]	1201	1202	1203	1204	1205	Mean	STDEV	Rel STDEV [%]
24	39.3	35.3	20.2	33.5	20.5	29.7	7.9	26.5
48	47.3	46.1	45.2	64.5	35.1	47.7	9.48	19.9
72	31.9	44.7	41.2	59.0	41.3	43.6	8.79	20.2

Appendix F Analysis of AOH metabolites in blood samples

Table 42 Metabolism of AOH: Dose group - 2000 mg / kg AOH; oral application, blood (GC-MS)

Mouse – Matrix: Blood										
AOH [ng / mL]										
t [h]	3101	3201	206	207	208	209*	210*	Mean	STDEV	Rel STDEV [%]
	male	female	male	male	male	male	male			
2	459	220						340	120	35
48			58.0	112	331	89	60	130	118	90.9

all animals

Mean	STDEV	Rel STDEV [%]
190	143	76

area -% peak area ratios of metabolites to IS were summed over all four metabolites and set in sum to 100%. The peak area ratios of the individual metabolites are accordingly indicated in % values.

Mouse – Matrix: Blood										
O3 = 10-hydroxy-AOH [area - %]										
t [h]	3101	3201	206	207	208	209*	210*	Mean	STDEV	Rel STDEV [%]
2	0.35	0.17						0.26	0.09	34.8
48			0.06	0.12	0.32	0.15	0.19	0.17	0.09	51.7

all animals

Mean	STDEV	Rel STDEV [%]
0.19	0.10	50

Mouse – Matrix: Blood										
O2 = 4-hydroxy-AOH [area - %]										
t [h]	3101	3201	206	207	208	209*	210*	Mean	STDEV	Rel STDEV [%]
2	0.20	0.11						0.16	0.05	28.9
48			0.04	0.05	0.11	0.04	0.04	0.07	0.03	44.3

all animals

Mean	STDEV	Rel STDEV [%]
0.10	0.06	57

Mouse – Matrix: Blood										
O1 = 8-hydroxy-AOH [area - %]										
t [h]	3101	3201	206	207	208	209*	210*	Mean	STDEV	Rel STDEV [%]
2	0.00	0.00						0.00	NA	NA
48			0.00	0.0	0.0	0.0	0.0	0.00	NA	NA

all animals

Mean	STDEV	Rel STDEV [%]
0.00	0.00	NA

Mouse – Matrix: Blood										
O4 = 2-hydroxy-AOH [area - %]										
t [h]	3101	3201	206	207	208	209*	210*	Mean	STDEV	Rel STDEV [%]
2	0.09	0.03						0.06	0.03	46.9
48			0.00	0.00	0.03	0.00	0.00	0.03	NA	NA

all animals

Mean	STDEV	Rel STDEV [%]
0.05	0.03	52

* Note: the additional animals 209 and 210 were older and heavier than the others.

Table 43 Calibration data AOH mice urine (GC-MS)

		AOH								Regression parameters		
		Nominal concentration [ng / mL]								y=a*x+b; no weighting		
Datum	**Matrix**	**250**	**500**	**750**	**1000**	**2000**	**4000**	**6000**		**Intercept**	**Slope**	**R²**
ID: 6												
20. Aug 14	mice urine	237	499	799	1015	1907	4062	5982	-			
Accuracy [%]	m/z 459	*95,0*	*99,7*	*106*	*101*	*95,3*	*102*	*99,7*	-	*-0,1610*	*1,81*	*0,999*
		50	**100**	**150**	**200**	**250**						
ID: 7												
22. Aug 14	mice urine	50,8	101	146	201	251	-	-	-			
Accuracy [%]	m/z 459	*102*	*101*	*97,2*	*101*	*100*	-	-	-	*-0,0754*	*2,04*	*0,999*

		AOH								Regression parameters		
		Nominal concentration [ng / mL]								y=a*x+b; no weighting		
Datum	**Matrix**	**10,00**	**25,00**	**50,00**	**75,00**	**100**				**Intercept**	**Slope**	**R²**
ID: 1												
28. Aug 14	mice blood	10,00	23,75	39.05*	75,80	96,72	-	-	-			
Accuracy [%]	m/z 459	*100*	*95,0*	*78,1*	*101*	*96,7*	-	-	-			
										-0,0144	*1,15*	*0,993*
28. Aug 14	mice blood	*11,60*	*25,82*	*44,18*	*78,95*	*102,5*	-	-	-			
Accuracy [%]	m/z 459	*116*	*103*	*88,4*	*105*	*103*	-	-	-			
* not included in regression												
		75,00	**100**	**250**	**500**							
ID: 2												
29. Aug 14	mice blood	73,37	103	252	463	-	-	-	-			
Accuracy [%]	m/z 459	*97,8*	*103*	*101*	*92,7*	-	-	-	-			
										0,0810	*0,915*	*0,989*
29. Aug 14	mice blood	69,41	95,75	263	531	-	-	-	-			
Accuracy [%]	m/z 459	*92,5*	*95,8*	*105*	*106*	-	-	-	-			

Appendix G Tentative calibration curves and estimation of actual concentration values of AOH and metabolites

Table 44 Estimation of actual concentration values (ng/mL) for AOH and oxidized metabolites. Note: only the AOH values are based on a validated calibration method, whereas the metabolite values must be considered as tentative and partially faulty (this applies in particular for the values of O3). The metabolites validations generally do not meet the required guideline standard due to lack of sample material.

Animal No.	Urine	g Urine	AOH ng/mL	AOH ng total	O3 ng/mL	O2 ng/mL	O1 ng/mL	O4 ng/mL	O3 ng total	O2 ng total	O1 ng total	O4 ng total
1101		**0,13**	39945,6	5192,9	2,17	5,26	2,56	21,20	0,28	0,68	0,33	2,76
1102		**0,18**	604,5	108,8	nn	2,69	nn	1,51	nn	0,48	nn	0,27
1103	24 h	1,52	446,8	679,1	nn	nn	nn	nn	nn	nn	nn	nn
1104		0,92	20501,3	18861,2	**140**	29,61	**714**	**131**	**129**	27,24	**657**	**121**
1105		**0,15**	4348,8	652,3	2,17	4,17	nn	9,64	0,33	0,63	nn	1,45
1201		2,49	9410,8	23432,9	4,75	5,71	nn	16,19	11,83	14,22	nn	40,31
1202		1,06	4879,4	5172,2	15,53	5,03	0,69	15,48	16,46	5,33	0,73	16,41
1203	24 h	0,73	328,4	239,7	12,71	2,72	nn	3,06	9,28	1,99	nn	2,23
1204		0,57	10090,4	5751,5	9,63	5,56	nn	13,43	5,49	3,17	nn	7,66
1205		0,60	29325,0	17595,0	93,68	13,95	**147**	43,60	56,21	8,37	**88**	26,16
1101		0,35	35895,0	12563,3	3,58	21,07	62,47	100,81	1,25	7,37	21,86	35,28
1102		0,39	4045,4	1577,7	nn	3,64	nn	14,00	nn	1,42	nn	5,46
1103	48 h	2,33	6222,9	14499,2	nn	5,34	nn	17,41	nn	12,44	nn	40,57
1104		1,07	2961,9	3169,2	4,55	4,27	nn	11,42	4,87	4,57	nn	12,22
1105		0,36	3022,4	1088,1	nn	4,02	nn	9,26	nn	1,45	nn	3,33
1201		0,50	1191,8	595,9	5,86	3,07	nn	7,33	2,93	1,54	nn	3,67
1202		1,05	1087,2	1141,5	2,88	3,06	nn	5,84	3,02	3,21	nn	6,13
1203	48 h	0,56	3821,7	2140,1	2,41	4,88	nn	15,04	1,35	2,73	nn	8,42
1204		0,98	2729,4	2674,8	1,91	2,86	nn	8,09	1,87	2,80	nn	7,93
1205		0,82	3010,9	2469,0	8,08	3,98	2,62	9,44	6,63	3,26	2,15	7,74
1101		0,87	6045,9	5259,9	13,76	7,00	3,72	26,90	11,97	6,09	3,24	23,40
1102		1,07	1331,7	1424,9	4,48	3,73	nn	5,26	4,79	3,99	nn	5,63
1103	72 h	1,98	616,2	1220,0	6,45	2,93	nn	3,97	12,77	5,80	nn	7,86
1104		1,79	1086,3	1944,5	5,30	3,64	6,65	7,89	9,49	6,52	11,90	14,12
1105		0,57	789,7	450,1	2,10	3,39	2,39	4,65	1,20	1,93	1,36	2,65
1201		0,78	102,3	79,8	16,22	2,59	nn	5,80	12,65	2,02	nn	4,52
1202		1,00	155,1	155,1	4,13	2,58	nn	3,62	4,13	2,58	nn	3,62
1203	72 h	0,39	446,9	174,3	5,80	2,76	nn	4,89	2,26	1,08	nn	1,91
1204		1,34	30,3	40,7	1,27	nn	nn	2,34	1,70	nn	nn	3,14
1205		1,48	22,8	33,8	1,64	nn	nn	1,74	2,43	nn	nn	2,58

O1 = 8-hydroxy-AOH; O2 = 4-hydroxy-AOH; O3 = 10-hydroxy-AOH; O4 = 2-hydroxy-AOH, values in bold are outside the calibration range

Calibration data of O1

LvLID	Amount (ratio)	Response (ratio)	bias (%)	Data File
1	0.0100	0.001134	-7.11	D:\Data\SG14\AOH\Blut\Snapshot\SG170902.D
2	0.0200	0.002095	8.82	D:\Data\SG14\AOH\Blut\Snapshot\SG170903.D
3	0.0500	0.003947	-2.33	D:\Data\SG14\AOH\Blut\SG170904.D
5	0.2000	0.014627	0.07	D:\Data\SG14\AOH\Blut\SG170906.D

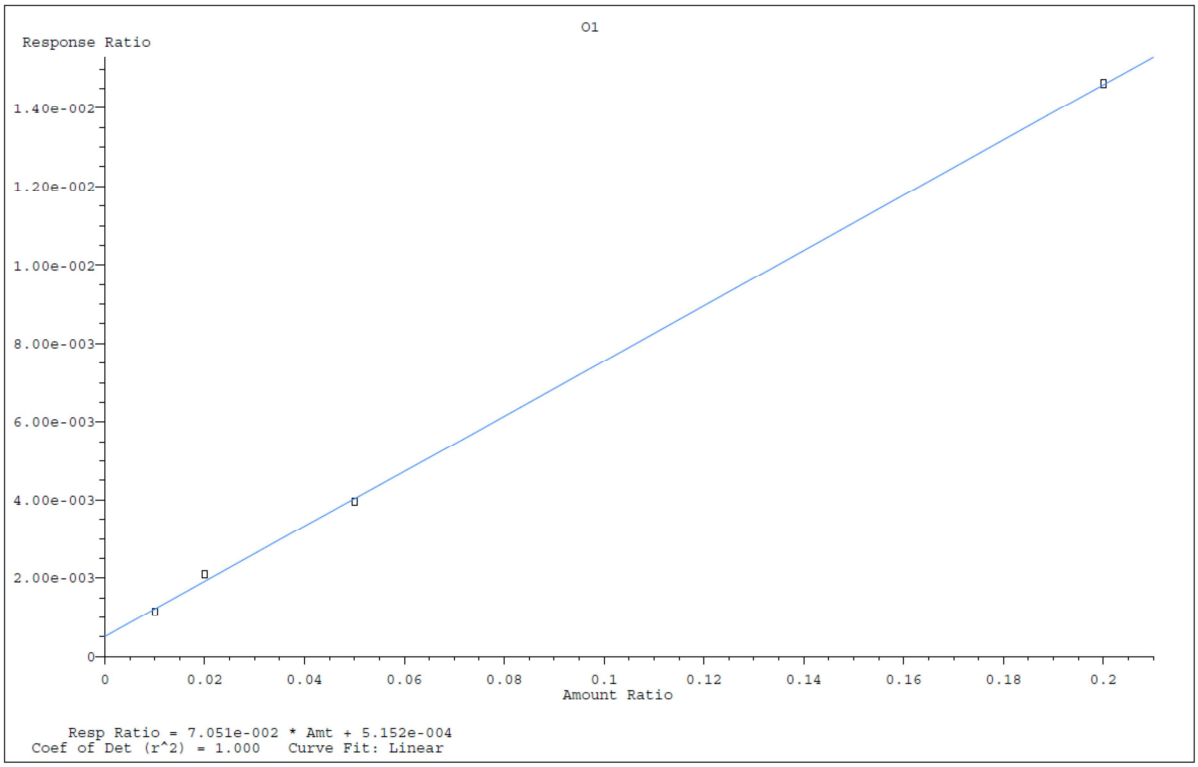

Figure 22 Tentative calibration curve (not guideline compliant) of 8-hydroxy-AOH (O1) used for the calculation in Table 44

Calibration data of O2

LvLID	Amount (ratio)	Response (ratio)	bias (%)	Data File
1	0.0100	0.015897	77.05	D:\Data\SG14\AOH\Blut\Snapshot\SG170902.D
2	0.0200	0.031743	17.50	D:\Data\SG14\AOH\Blut\Snapshot\SG170903.D
3	0.0500	0.063430	-21.81	D:\Data\SG14\AOH\Blut\SG170904.D
4	0.1000	0.176194	2.85	D:\Data\SG14\AOH\Blut\SG170905.D
5	0.2000	0.352830	0.33	D:\Data\SG14\AOH\Blut\SG170906.D

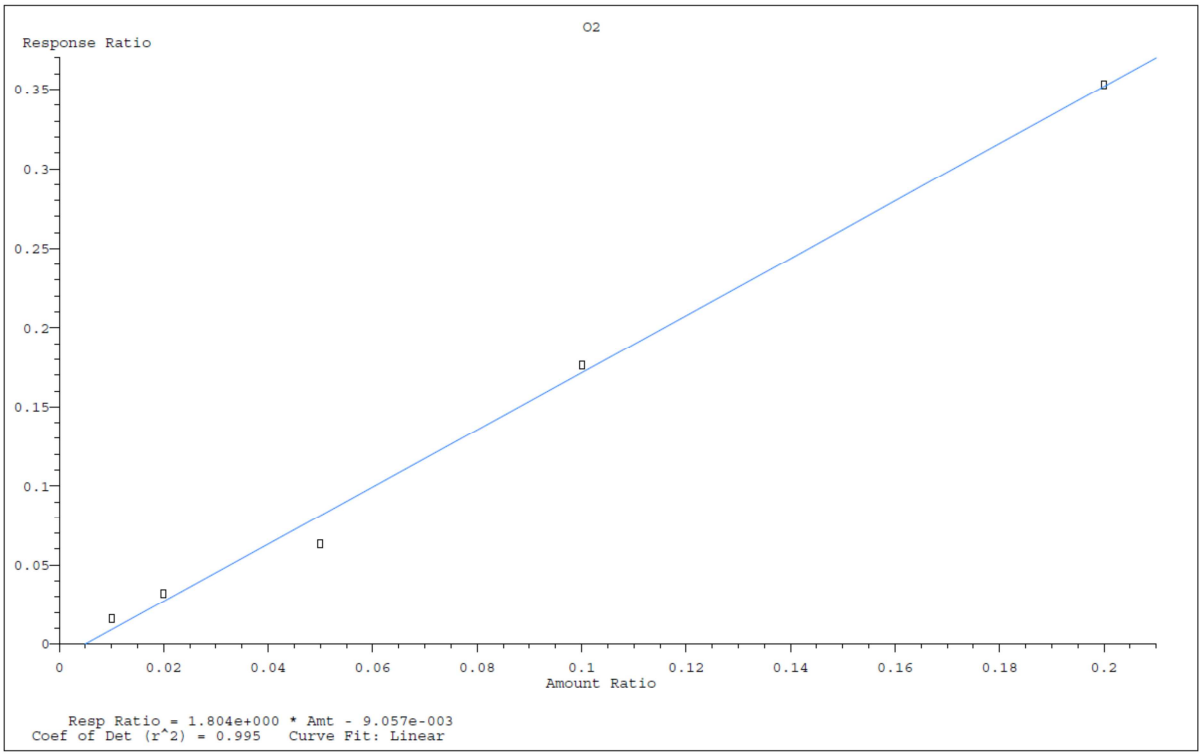

Figure 23 Tentative calibration curve (not guideline compliant) of 4-hydroxy-AOH (O2) used for the calculation in Table 44

Calibration data of O3

LvLID	Amount (ratio)	Response (ratio)	bias (%)	Data File
1	0.0100	0.002442	17.13	D:\Data\SG14\AOH\Blut\Snapshot\SG170902.D
2	0.0200	0.004002	3.40	D:\Data\SG14\AOH\Blut\Snapshot\SG170903.D
3	0.0500	0.007585	-17.82	D:\Data\SG14\AOH\Blut\SG170904.D
4	0.1000	0.019711	8.54	D:\Data\SG14\AOH\Blut\SG170905.D
5	0.2000	0.035626	-1.10	D:\Data\SG14\AOH\Blut\SG170906.D

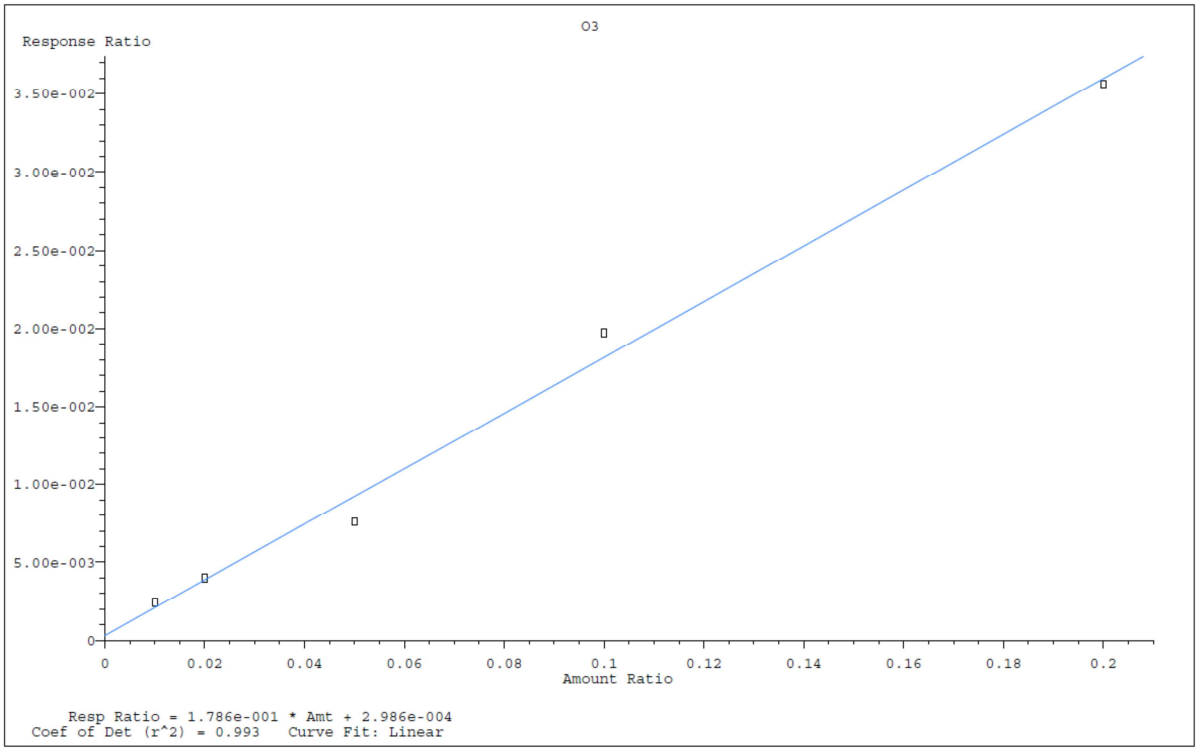

Figure 24 Tentative calibration curve (not guideline compliant) of 10-hydroxy-AOH (O3) used for the calculation in Table 44. Here there is a very large uncertainty in the values as the concentration of the calibration solutions may be incorrect.

Calibration data of O4

LvLID	Amount (ratio)	Response (ratio)	bias (%)	Data File
1	0.0100	0.003156	27.02	D:\Data\SG14\AOH\Blut\Snapshot\SG170902.D
2	0.0200	0.005398	1.65	D:\Data\SG14\AOH\Blut\Snapshot\SG170903.D
3	0.0500	0.010894	-20.98	D:\Data\SG14\AOH\Blut\SG170904.D
4	0.1000	0.030815	10.40	D:\Data\SG14\AOH\Blut\SG170905.D
5	0.2000	0.055393	-1.37	D:\Data\SG14\AOH\Blut\SG170906.D

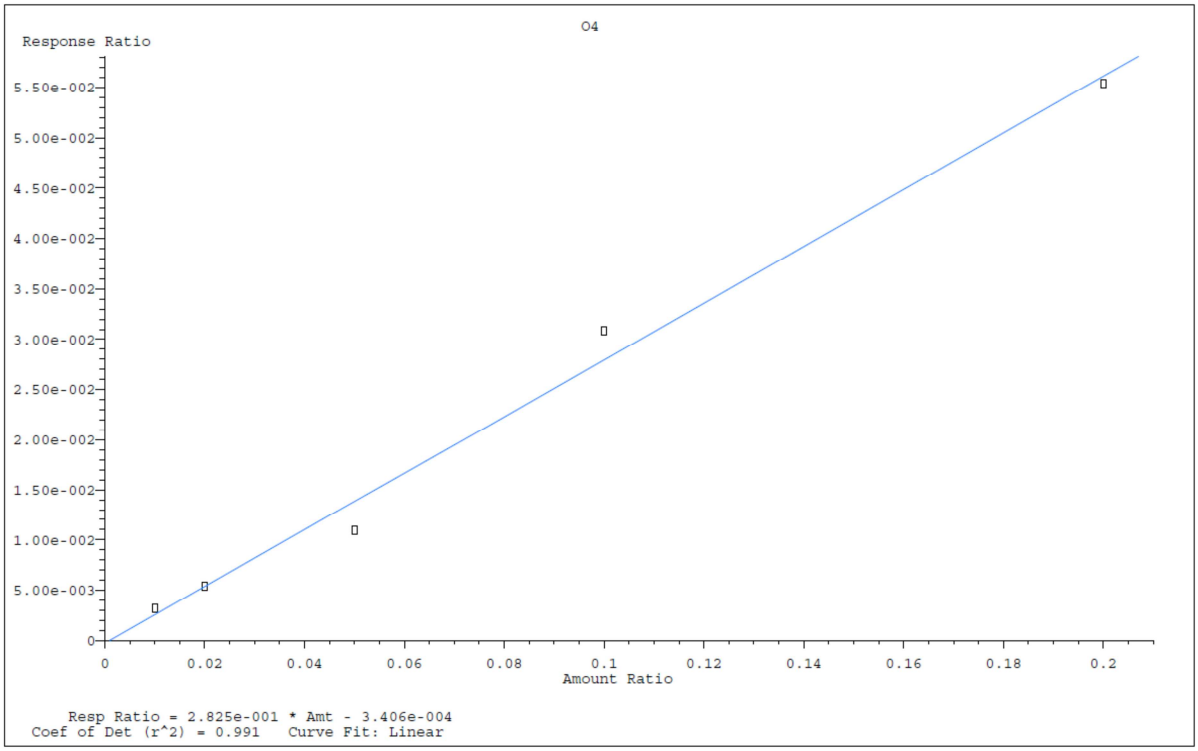

Figure 25 Tentative calibration curve (not guideline compliant) of 2-hydroxy-AOH (O4) used for the calculation in Table 44

Appendix H Determination of AOH in mice whole blood and urine

Fraunhofer ITEM

Validation Report

Original 1 of 2

Determination of AOH in mice whole blood and urine

Appendix H of the EXTERNAL SCIENTIFIC REPORT:

Combined toxicokinetic and *in vivo* genotoxicity study on Alternaria toxins

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This appended validation report consists of 41 pages.

Date: 03.10.2014

Abbreviations

AOH	Alternariol
aq	aqueous
AS	Autosampler
BLQ	Below Limit of Quantification
BSTFA	N,O-Bis-(trimethylsilyl)trifluoroacetamide
CAD	Collision Associated Dissociation
Cal	Calibration
Conc.	Concentration
C.V.	Coefficient of Variation
CXP	Cell Exit Potential
DMSO	Dimethylsulfoxide
DP	Declustering Potential
EI	Electron Impact
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
K3EDTA	Potassium Ethylendiamintetraacetate
LC	Liquid Chromatography
LLOQ	Lower Limit of Quantification
MF	Matrix Factor
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry / Spectrometer
MS/MS	Tandem Mass Spectrometry
NA	Not Applicable
Q1	Quadrupole 1
Q3	Quadrupole 3
QC	Quality Control
QCL	QCLow
QCM	QCMedium
QCH	QCHigh

SD	Standard Deviation; Population Standard Deviation
SN	Serial Number
rcf	relative centrifugal force
ULQ	Upper Limit of Quantification

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1 Statement of the person responsible for the validation

Fraunhofer ITEM

Associated with the study: EFSA EXTERNAL SCIENTIFIC REPORT:

"Combined toxicokinetic and *in vivo* genotoxicity study on *Alternaria* toxins"

Test item: Alternariol （AOH）

Title: Determination of AOH in mice whole blood and urine

This is a non GLP validation. However, the principles of GLP as well as the EMA-"Guideline on bioanalytical method validation"[1] were taken into consideration and this validation was conducted in the spirit of GLP.

Date

03.10.2014

……

Signature

……

Fraunhofer Institute for Toxicology and Experimental Medicine ITEM

Person responsible for the validation

Dr. S. Schuchardt

Objective:

1. Validation of a LC-MS/MS method for the quantification of AOH in mice whole blood.

2. Validation of a GC-MS method for the quantification of AOH in mice urine.

3. Monitoring of AOH and selected hydroxyl metabolites in mice urine and whole blood by GC-MS analysis.

Fraunhofer ITEM

Associated with the study:

EFSA EXTERNAL SCIENTIFIC REPORT: "Combined toxicokinetic and *in vivo* genotoxicity study on *Alternaria* toxins"

Sponsor:

European Food Safety Authority (EFSA)

Via Carlo Magno 1A

43126 Parma (PR)

ITALY

Test facility:

Fraunhofer ITEM, Nikolai-Fuchs-Strasse 1

30625 Hannover, Germany

Personnel:

Person responsible for the validation: S. Schuchardt

Laboratory Managers:	S. Schuchardt
	K. Bluemlein
Scientists responsible for the report:	K. Bluemlein
	S. Schuchardt
Technicians:	U. Sänger
	S. Gerling

Distribution of this report:

Sponsor:	1 original
Person responsible for the validation:	1 original

2 Summary

This report describes the validation of the determination of alternariol (AOH):

1. In mice whole blood stabilised with potassium ethylendiamintetraacetate (K3EDTA) by liquid chromatography tandem mass spectrometry (LC-MS/MS)
   and
2. in mice urine by gas chromatography - mass spectrometry (GC-MS).

The GC-MS method developed for the determination of AOH in urine will also allow the semi-quantitative determination (chapter 5) of the four alternariol metabolites 8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH and 2-hydroxy-AOH (**Fig. 1**).

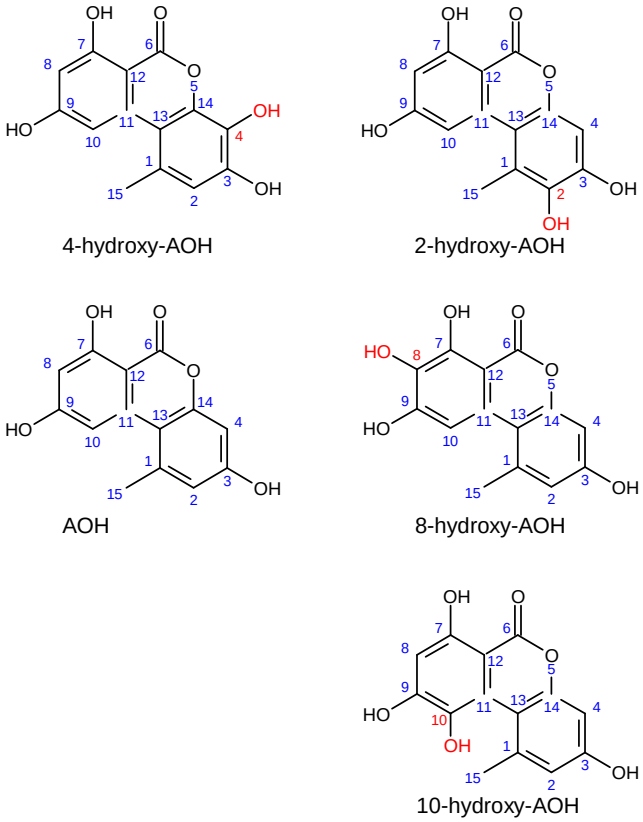


Fig. 1: Alternariol (AOH) and the monohydroxylated metabolites 8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH and 2-hydroxy-AOH.

Those four metabolites, which each carry a hydroxyl group at one of the two aromatic rings, were already described in detail by N. H. Schebb in his diploma thesis [2]. Schebb's peak assignment of the individual metabolites in GC-MS analysis was successfully confirmed in the associated Fraunhofer ITEM study applying NMR[1] spectroscopy and mass spectrometry (MS) for identification. The validation of a quantitative method for all four metabolites in mice urine and whole blood had to be omitted due to a lack of sample standards.

[1] nuclear magnetic resonance

2.1 Determination of AOH in mice whole blood by LC-MS/MS analysis

In order to correct for e.g. analyte loss during sample preparation or instrumental drift 7-Hydroxy-4-phenylcoumarin (HPC) was used as internal standard (IS). Quantitation was achieved by running the mass spectrometer (MS) in multiple reaction monitoring (MRM) mode. The MRM transitions (mass to charge ratios, m/z) used for the quantitation was 257 to 213 for the analyte AOH and 237 to 193 for the internal standard HPC. The selected MRM traced were not affected by interferences (chapter 4.2.1).

The method described here, provides a linear correlation between AOH concentration and instrument response over the investigated concentration range of 11 to 774 ng / mL (chapter 4.3.1).

The determined accuracy values for the calibration standards (chapter 4.3.1) did comply with the EMA guideline[1]. Precision and accuracy data obtained for the QC standards were conforming to the EMA guideline[1] (chapter 4.5.1). For neither the analyte AOH nor the IS HPC significant carry over was observed (chapter 4.4.1). The stability of AOH working solutions at approx. 4 – 8 °C was assessed over a time period of 30 days. The respective data demonstrate stability over the investigated time period (chapter 4.5.3). Analyte integrity in mice whole blood extracts during autosampler storage at 7 °C as well as under uncontrolled temperature conditions was investigated and confirmed over 30 and 27 h respectively (chapter 4.6.1).

2.2 Determination of AOH in mice urine by GC-MS analysis

AOH and its metabolites (**Fig. 1**) were monitored by GC-MS analysis after their derivatisation with N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA).

In order to correct for e.g. analyte loss during sample preparation or instrumental drift 6, 7- Dihydroxycoumarin (DHC) was used as internal standard (IS). Quantitation of AOH and monitoring of its hydroxyl metabolites was achieved by

Single Ion Monitoring (SIM). The analyte AOH was monitored on the mass to charge ratios (m/z) 459 (quantifier) and 222 (qualifier) and the internal standard DHC on the m/z trace 322. The SIM traces m/z 459 and 322 were not affected by interferences (chapter 4.2.2). All four metabolites (8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH and 2-hydroxy-AOH) were monitored on m/z 547.

The method described here, provides a linear correlation between AOH concentration and instrument response over the investigated concentration range of 50 to 6000 ng / mL (chapter 4.3.2).

The determined accuracy values for the calibration standards (chapter 4.3.2) did comply with the EMA guideline[1]. Precision and accuracy data obtained for the QC standards were conforming to the EMA guideline[1] (chapter 4.5.2). For neither the analyte AOH nor the IS HPC significant carry over was observed (chapter 4.4.2). The stability of AOH working solutions at approx. 4 – 8 °C was assessed over a time period of 30 days. The respective data demonstrate stability over the investigated time period (chapter 4.5.3). Analyte integrity in mice urine extracts during autosampler storage was omitted, due to the nature of the scheduled batch analysis of the study samples (chapter 4.6.2).

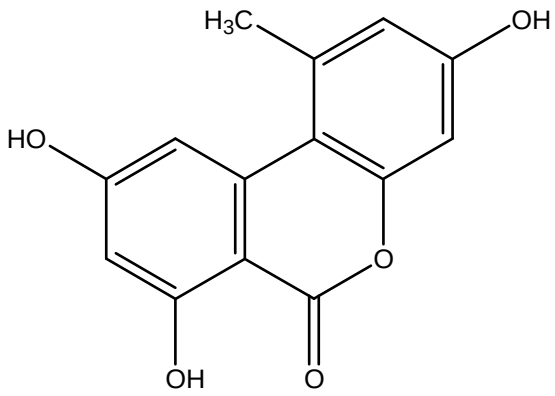
3 Materials and Methods

3.1 Materials

3.1.1 Analyte

Name:	Alternariol （AOH）
CAS no.:	641-38-3
Batch no.:	0435215-2
Purity:	≥ 98 %
Expiration date:	18th December 2014
Supplier:	Cayman Chemical
Molecular formula:	$C_{14}H_{10}O_5$
Molecular weight:	258.23 g / mol
Certificate of Analysis:	Not available

Structure:



3.1.2 Internal Standard 1 – LC-MS/MS analysis

Product name:	7-Hydroxy-4-phenylcoumarin (HPC)
CAS no.:	2555-30-8
Batch no.:	18203MAV
Expiration date:	June 2019
Supplier:	Sigma
Molecular formula:	$C_{15}H_{10}O_3$
Molecular weight:	238.24 g / mol
Certificate of Analysis:	Not available

3.1.3 Internal Standard 2 – GC-MS analysis

Product name:	6, 7- Dihydroxycoumarin (DHC)
CAS no.:	305-01-1
Batch no.:	STBD1992V
Expiration date:	04th March 2019
Supplier:	Sigma
Molecular formula:	$C_9H_6O_4$
Molecular weight:	178.14 g / mol
Certificate of Analysis:	Not available

3.1.4 Chemicals

Zinc sulphate heptahydrate (Sigma Aldrich)

Methanol, gradient grade for HPLC (Acros Organics)

Isopropanol, for gas chromatography (Acros Organics)

Acetonitrile, Baker Analysed LC-MS Reagent (J. T. Baker)

Formic acid, eluent additive for LC-MS (Sigma Aldrich)

Methanol, LC-MS reagent (J. T. Baker)

Water, LC-MS Reagent (J. T. Baker)

Dimethylsulfoxide (Sigma Aldrich)

Ethylacetate, Supra Solv for GC (Merck)

N,O-Bis-(trimethylsilyl)trifluoroacetamide (Supleco, 99.5%)

3.1.5 **Reagents**

LC-MS/MS Analysis

Zinc sulphate solution (0.1 M)

Zinc sulphate heptahydrate (0.75 g) was dissolved in water (25 mL) to give a nominal concentration of 0.03 g / mL (0.1 M).

Methanolic zinc sulphate solution – Precipitating reagent

Zinc sulphate solution (0.1 M) was diluted with methanol 1:3 (e.g. 5 mL zinc sulphate solution : 15 mL methanol).

50% Methanol (aq)

Aqueous methanol solution was prepared by mixing water and methanol in a ratio of 1:1.

70 % Methanol (aq)

Aqueous methanol solution was prepared by mixing water and methanol in a ratio of 30 : 70.

Isocractic eluent – Trap column

The iscocratic eluent was prepared by mixing methanol and water in a ratio of 25 : 75.

Needle wash (0.1 % formic acid (aq) : isopropanol : methanol : acetonitrile 1:1:1:1)

The needle wash solution consisted of 0.1 % formic acid (aq), isopropanol, methanol and acetonitrile in the ratios 1:1:1:1.

3.1.6 **Matrix**

Mice whole blood (K3EDTA, Research for Health, ITEM)

Mice urine (Research for Health, ITEM)

3.2 **Analytical Methods**

3.2.1 **General Comments**

The quantification of AOH in mice whole blood was achieved by LC-MS/MS analysis in MRM mode using matrix matched standards and 7-Hydroxy-4-phenylcoumarine (HPC) as internal standard.

The quantification of AOH in mice urine was achieved by GC-MS analysis in SIM mode using matrix matched standards and 6,7-Dihydroxycoumarin (DHC) as internal standard.

Due to a lack of standards for the AOH metabolites 8-hydroxy-AOH; 4-hydroxy-AOH; 10-hydroxy-AOH and 2-hydroxy-AOH no quantitative data can be obtained and validation for those four compounds was omitted. Microsomal incubation of AOH was used to obtain an AOH-metabolite mix of unknown concentration, which was used to assign metabolite IDs in GC-MS analysis (chapter 5).

3.2.2 Instruments

HPLC Agilent 1260 consisting of:

- o Degasser
- o Binary Pump
- o Isocratic Pump
- o Autosampler
- o Thermostat
- o Temperature Controlled Compartment

MS (QTrap 5500, ABSCIEX)

GC HP 6890 (GC) / HP 5973 (MS); Gerstel MPS 2 Autosampler

Balance (Sartorius RC210S-OD1)

Centrifuge (Sigma 1-1S)

Fridge (approx. 4 - 8 °C)

Freezer (approx. -20 °C)

MS2 Minishaker (IKA)

Cold room (7 – 12 °C)

Evaporation unit (Supelco)

3.2.3 Materials

Amber glass autosampler vials (2 mL)

Clear glass autosampler vials (2 mL)

Polypropylene vials (different sizes)

Eppendorf tubes (0.5 and 2 mL)

Pipettes (different sizes, Gilson, Brandt and Eppendorf)

3.2.4 Instrument Parameters

The instrument parameters and settings for the determination of AOH in mice whole blood by LC-MS/MS parameter are listed in **Tab. 1** and **2.**

The determination of AOH and the monitoring of its four metabolites 8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH and 2-hydroxy-AOH in mice urine was achieved by GC-MS. The respective parameters and settings are listed in **Tab. 3.**

Tab. 1: HPLC parameters and settings for AOH analysis in mice whole blood

Parameter	**Settings**
System	Agilent 1260
Trap column	Zorbax Eclipse Plus C8, 2.1x12.5 mm, 5 µm ; SN : USUQB03701
Analytical column	Poroshell 120 EC-C18, 3x50 mm, 2.7 µm; SN: USCFZ12555
Column temperature [°C]	50
Injection volume [µL]	10
Autosampler temperature [°C]	7 or uncontrolled
Needle wash	0.1% formic acid (aq) :acetonitrile:methanol:isopropanol (1:1:1:1)
Mobile phase	
Trap column	Isocratic: methanol / water 25:75
Analytical column	**A:** water
	B: methanol

Gradients			
Trap column			
Time [min]	Flow rate [µL / min]	Valve position	time [min]
0.00	500	right	0.00
0.01	500	left	0.01
1.51	500	left	1.52
1.52	500	right	1.53
22.00	500	right	18.00
		left	18.01
		left	22.10
		right	22.20

Analytical column		
Time [min]	Flow rate [µL / min]	B [%]
0.00	500	25
1.00	500	25
10.0	500	80
15.00	500	80
15.01	500	95
18.00	500	95
18.01	500	25
22.00	500	25

Tab. 2: MS parameters and settings for AOH analysis in mice whole blood

Parameters	**Settings**
System	ABSCIEX QTRAP 5500
Source	Turbo V™
Mode	Negative
Capillary voltage [V]	-4500
Curtain gas (N_2) [psi]	30
Gas 1 (N_2) [psi]	50
Gas 2 (N_2) [psi]	70
Temperature [°C]	550
CAD gas	Medium
Dwell time [ms]	150
Entrance potential [V]	-10

MRM transitions

Compound	Q1 m/z	Q3 m/z	Collision energy	CXP	DP
AOH - Quantifier	257	213	-36V	-15V	-60V
AOH – Qualifier	257	147	-46V	-5V	-60V
HPC – Quantifier	237	193	-35V	-15V	-50V

Tab. 3: GC-MS parameters and settings for AOH analysis in mice urine

Parameter	Settings
System	HP 6890 (GC) / HP 5973 (MS); Gerstel MPS 2 Autosampler
Mode	Electron impact (EI)
Ionisation energy [eV]	70
Column	HP5ms, length: 30m; diameter: 0.25 mm; film thickness: 0.25 µm
Carrier gas	Helium
Carrier gas flow [mL / min]	1.2 (constant flow)
Injection volume [µL]	1
Injector temperature [°C]	250
Injector mode	splitless
Temperature program	Start: 60°C for 1 min; End: 290°C for 15 min; Rate: 15°C / min
Quadrupole temperature [°C]	150
Data acquisition	
Compound ID	Target (m/z)
AOH	459 (qualifier: 222)
DHC (IS)	322
8-hydroxy-AOH	547
4-hydroxy-AOH	547
10-hydroxy-AOH	547
2-hydroxy-AOH	547

3.3 Calibration and Quality Control Standards

3.3.1 Preparation of analyte stock solution

Alternariol (1 mg) was dissolved in DMSO (310 µL) to give a stock solution with a nominal concentration of 3.23 mg / mL. This stock solution was stored at approx. – 20 °C.

3.3.2 Preparation of intermediate and analyte working solution

3.3.2.1 Solutions for LC-MS/MS analysis

Intermediate solution A – LC-MS/MS analysis

The AOH stock solution was diluted 1:300 in methanol. The resulting intermediate solution A had a nominal AOH concentration of 10.75 µg / mL. This solution was stored at approx. 4 – 8 °C.

Standard working solutions – LC-MS/MS analysis

The AOH intermediate solution A was diluted in methanol to give nine standard working solutions with the following nominal concentrations: 53.8; 135; 269; 430; 538; 1344; 2150; 2688 and 3870 ng / mL.

Quality Control working solutions – LC-MS/MS analysis

The AOH intermediate solution A was diluted in methanol to give three quality control working solutions QCL, QCM and QCH. The concentrations of those three solutions did cover the lower, the mid and the high end of the standard working solution.

3.3.2.2 Solutions for GC-MS analysis

Intermediate solution A – GC-MS/MS analysis

The AOH stock solution (3.1 µL) was diluted in methanol (996.9 µL). The resulting intermediate solution A had a nominal AOH concentration of 10.0 µg / mL. This solution was stored at approx. 4 – 8 °C.

Intermediate solution B – GC-MS/MS analysis

The AOH intermediate solution B (100 µL) was diluted in methanol / water 70:30 (900 µL). The resulting intermediate solution B had a nominal AOH concentration of 1.00 µg / mL. This solution was stored at approx. 4 – 8 °C.

3.3.3 Preparation of IS stock solution

HPC – LC-MS/MS

HPC (4.79 mg) were dissolved in methanol (10 mL) resulting in an IS stock solution of a HPC concentration of 0.48 mg / mL.

DHC – GC-MS

DHC (5.10 mg) were dissolved in methanol (5 mL) resulting in an IS stock solution of a DHC concentration of 1.02 mg / mL.

3.3.4 Preparation of IS working solution

HPC - IS working solution A – LC-MS/MS

An HPC stock solution was prepared in methanol / water 70:30 with a nominal concentration of 10 µg / mL.

HPC - IS working solution B – LC-MS/MS

HPC-IS working solution A (375 µL) was diluted in methanol / water 70 : 30 (9.625 mL) to give HPC-IS working solution B with a nominal concentration of 375 ng / mL.

DHC - IS working solution A – GC-MS

DHC IS stock solution (49 µL) were transferred into a 10 mL measuring flask. The flask was filled to the mark with methanol / water 70:30 to give a working solution A with a nominal concentration of 5 µg / mL.

3.3.5 Blank and Zero Samples

LC-MS/MS analysis

Blank

K3EDTA mice whole blood (25 µL) was transferred into a 0.5-mL Eppendorf tube. Methanol (11 µL) was added and the sample mixed on a vortex mixer. Proteins were precipitated by adding methanolic zinc sulphate solution (250 µL). The solution was mixed on a vortex mixer for 30 sec and then centrifuged at 10000 rcf for 10 min at room temperature. Methanol (100 µL) was added, the sample mixed on a vortex mixer and then centrifuged at 10000 rcf for 5 min at room temperature. An aliquot (100 µL) of the supernatant was transferred into a 2-mL HPLC-vial, aqueous methanol solution (400 µL, 50 % methanol (aq)) was added and the sample mixed on a vortex mixer prior to LC-MS/MS analysis.

Zero

K3EDTA mice whole blood (25 µL) was transferred into a 0.5-mL Eppendorf tube. IS intermediate solution B (6 µL) and methanol (5 µL) were added and the sample mixed on a vortex mixer. Proteins were precipitated by adding methanolic zinc sulphate solution (250 µL). The solution was mixed on a vortex mixer for 30 sec and then centrifuged at 10000 rcf for 10 min at room temperature. Methanol (100 µL) was added, the sample mixed on a vortex mixer and then centrifuged at 10000 rcf for 5 min at room temperature. An aliquot (100 µL) of the supernatant was transferred into a 2-mL HPLC-vial, aqueous methanol solution (400 µL, 50 % methanol (aq)) was added and the sample mixed on a vortex mixer prior to LC-MS/MS analysis.

GC-MS analysis

Blank

Mice urine (200 µL) was transferred into a 2-mL Eppendorf tube. Ethylacetate (300 µL) were added and the sample mixed on a vortex mixer. After

centrifugation (8000 rcf; 2 min, room temperature) the supernatant was transferred into a clear 2-mL autosampler vial. The residue was extracted with ethylacetate (300 µL) by thorough shaking using a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was combined with the first supernatant and the resulting sample was dried in a nitrogen stream. After complete removal of all solvent and water, BSTFA (40 µL) was added. The resulting blank was left over night at room temperature, to allow complete derivatisation, prior to GC-MS analysis.

Zero

IS intermediate solution A (20 µL) and mice urine (200 µL) were transferred into a 2-mL Eppendorf tube. Ethylacetate (300 µL) was added and the sample mixed on a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was transferred into a clear 2-mL autosampler vial. The residue was extracted with ethylacetate (300 µL) by thorough shaking using a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was combined with the first supernatant and the resulting sample was dried in a nitrogen stream. After complete removal of all solvent and water, BSTFA (40 µL) was added. The resulting zero was left over night at room temperature, to allow complete derivatisation, prior to GC-MS analysis.

3.3.6 **Preparation of Calibration Standards**

LC-MS/MS - analysis

Calibrations standards were prepared by spiking mice whole blood aliquots (25 µL) with the respective standard working solution (5 µL) and IS working solution B (6 µL). The samples were then mixed on a vortex mixer. Proteins were precipitated by adding methanolic zinc sulphate solution (250 µL). The solution was mixed on a vortex mixer for 30 sec and then centrifuged at 10000 rcf for 10 min at room temperature. Methanol (100 µL) was added, the sample mixed on a vortex mixer and then centrifuged at 10000 rcf for 5 min at

room temperature. An aliquot (100 µL) of the supernatant was transferred into a 2-mL HPLC-vial, aqueous methanol solution (400 µL, 50 % methanol (aq)) was added and the sample mixed on a vortex mixer prior to LC-MS/MS analysis. The nominal AOH concentration of those calibration standards were 11; 27; 54; 86; 108; 269; 430; 538 and 774 ng / mL.

GC-MS – analysis

Calibration standards were prepared by adding aliquots of either AOH intermediate solution A or B, and IS intermediate solution A (20 µL) and mice urine (200 µL) into a 2-mL Eppendorf tube. Ethylacetate (300 µL) was added and the sample mixed on a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was transferred into a clear 2-mL autosampler vial. The residue was extracted with ethylacetate (300 µL) by thorough shaking using a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was combined with the first supernatant and the resulting sample was dried in a nitrogen stream. After complete removal of all solvent and water, BSTFA (40 µL) was added. The resulting calibration standards were left over night at room temperature, to allow complete derivatisation, prior to GC-MS analysis. The nominal AOH concentration of those calibration standards were 250; 500; 750; 1000; 2000; 4000 and 6000 ng / mL.

3.3.7 **Preparation of Quality Control Standards**

LC-MS/MS - analysis

Quality control standards were prepared by spiking mice whole blood aliquots (25 µL) with the respective standard working solution (5 µL) and IS working solution B (6 µL). The samples were then mixed on a vortex mixer. Proteins were precipitated by adding methanolic zinc sulphate solution (250 µL). The solution was mixed on a vortex mixer for 30 sec and then centrifuged at 10000 rcf for 10 min at room temperature. Methanol (100 µL) was added, the

sample mixed on a vortex mixer and then centrifuged at 10000 rcf for 5 min at room temperature. An aliquot (100 µL) of the supernatant was transferred into a 2-mL HPLC-vial, aqueous methanol solution (400 µL, 50 % methanol (aq)) was added and the sample mixed on a vortex mixer prior to LC-MS/MS analysis. This resulted in quality control standards QCL, QCM and QCH did cover the concentration range defined by the calibration standards.

GC-MS – analysis

Quality control standards were prepared by adding aliquots of either AOH intermediate solution A or B, and IS intermediate solution A (20 µL) and mice urine (200 µL) into a 2-mL Eppendorf tube. Ethylacetate (300 µL) was added and the sample mixed on a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was transferred into a clear 2-mL autosampler vial. The residue was extracted with ethylacetate (300 µL) by thorough shaking using a vortex mixer. After centrifugation (8000 rcf; 2 min, room temperature) the supernatant was combined with the first supernatant and the resulting sample was dried in a nitrogen stream. After complete removal of all solvent and water, BSTFA (40 µL) was added. The resulting quality control standards were left over night at room temperature, to allow complete derivatisation, prior to GC-MS analysis. The nominal AOH concentration of those quality control standards were 500; 1000 and 4000 ng / mL.

4 Analysis and Results

4.1 Run Acceptance Criteria

The run acceptance criteria applied throughout this validation are based on the EMA guideline[1]:

- The back calculated concentrations of calibration and quality control standards should be within ± 15 % of their nominal concentration. For the

LLOQ standard the accuracy should be within ± 20 % of its nominal concentration.

- 75 % of the calibration standards should fulfil the above accuracy requirement.
- 67 % of the QC standards and 50 % of each QC concentration level should fulfil the above accuracy requirement.

In this report, all standards not meeting the EMA guideline[1] criteria are highlighted in red.

4.2 Selectivity - AOH

4.2.1 LC-MS/MS - mice whole blood

Analyte selectivity was investigated by preparing 6 blank and 6 LLOQ samples from pooled mice whole blood. The average response for the analyte AOH in the blank samples was determined as 478 counts (**Tab. 4**). This equals 1 % of the LLOQ average value of 45840 counts (**Tab. 4**). The variation of the analyte peak areas between the individual blank samples was with 111 % fairly high. The highest analyte peak area detected in the six blank samples was 1410 counts which equals 3 % of the LLOQ average value of 45840. This demonstrates that despite the high variation, no significant interference was present for the analyte AOH on the chosen MRM-trace at the respective retention time window.

In none of the blank samples an IS signal was detected (**Tab. 4**).

Tab. 4: Selectivity data for AOH in mice whole blood.

#	Sample Name	Sample ID	Analyte peak area [counts]	IS peak area [counts]
1	DoBl200814-01	blank_01	0	0
2	DoBl200814-02	blank_02	702	0
3	DoBl200814-03	blank_03	1410	0
4	DoBl200814-04	blank_04	0	0
5	DoBl200814-05	blank_05	0	0
6	DoBl200814-06	blank_06	754	0
		Mean value (n = 6)	**478**	
		STDEV	**529**	
		C. V. [%]	**111**	
7	QC 300714-01	LLOQ_01	50100	830000
8	QC 300714-02	LLOQ_02	46800	859000
9	QC 300714-03	LLOQ_03	44500	812000
10	QC 300714-04	LLOQ_04	45900	827000
11	QC 300714-05	LLOQ_05	41900	819000
		Mean value (n = 5)	**45840**	**829400**
		STDEV	**2698**	**16082**
		C. V. [%]	**5.89**	**1.94**

4.2.2 GC-MS - mice urine

Analyte selectivity was investigated by preparing 5 blank and 5 LLOQ samples from pooled mice urine. The average response for the analyte AOH in the blank samples was determined as 8895 counts (**Tab. 5**). This equals 0.3 % of the LLOQ average value of 3543509 counts (**Tab. 5**). The variation of the analyte peak areas between the individual blank samples was with 39.5 % fairly high. The

highest analyte peak area detected in the five blank samples was 15963 counts which equals 0.5 % of the LLOQ average value of 3543509. This demonstrates that despite the high variation, the matrix contribution to the analyte signal was not significant.

Tab. 5: Selectivity data for AOH in mice whole blood.

#	Sample Name	Sample ID	Analyte peak areas [counts]	IS peak area [counts]
1	SG220801	DB 210814-01	15963	17730
2	SG220826	DB 210814-02	9246	15461
3	SG210801	DB 200814-01	7960	16733
4	SG200802	DB 190814-01	7352	17559
5	SG200828	DB 190814-02	3953	19837
		Mean value (n = 5)	**8895**	**17464**
		STDEV	**3509**	**929**
		C.V. [%]	**39.5**	**5.32**
6	SG220803	QC L 210814-01	3556260	2140782
7	SG220817	QC L 210814-02	3602904	2083234
8	SG210803	QC L 200814-01	3542263	2208021
9	SG210817	QC L 200814-02	3288888	2235380
10	SG200824	QC L 190814-01	3727228	2163014
		Mean value (n = 5)	**3543509**	**2166086**
		STDEV	**143022**	**53055**
		C.V. [%]	**4.0**	**2.45**

4.3 Calibration curve and LLOQ - AOH

4.3.1 LC-MS/MS - mice whole blood

For the analyte AOH a concentration range between 11 to 774 ng / mL was validated in mice whole blood matrix. A linear relationship between concentration and signal response was found. In all cases a linear regression was applied (**Fig. 2** and **Tab. 6**), with the LLOQ being 11 ng / mL.

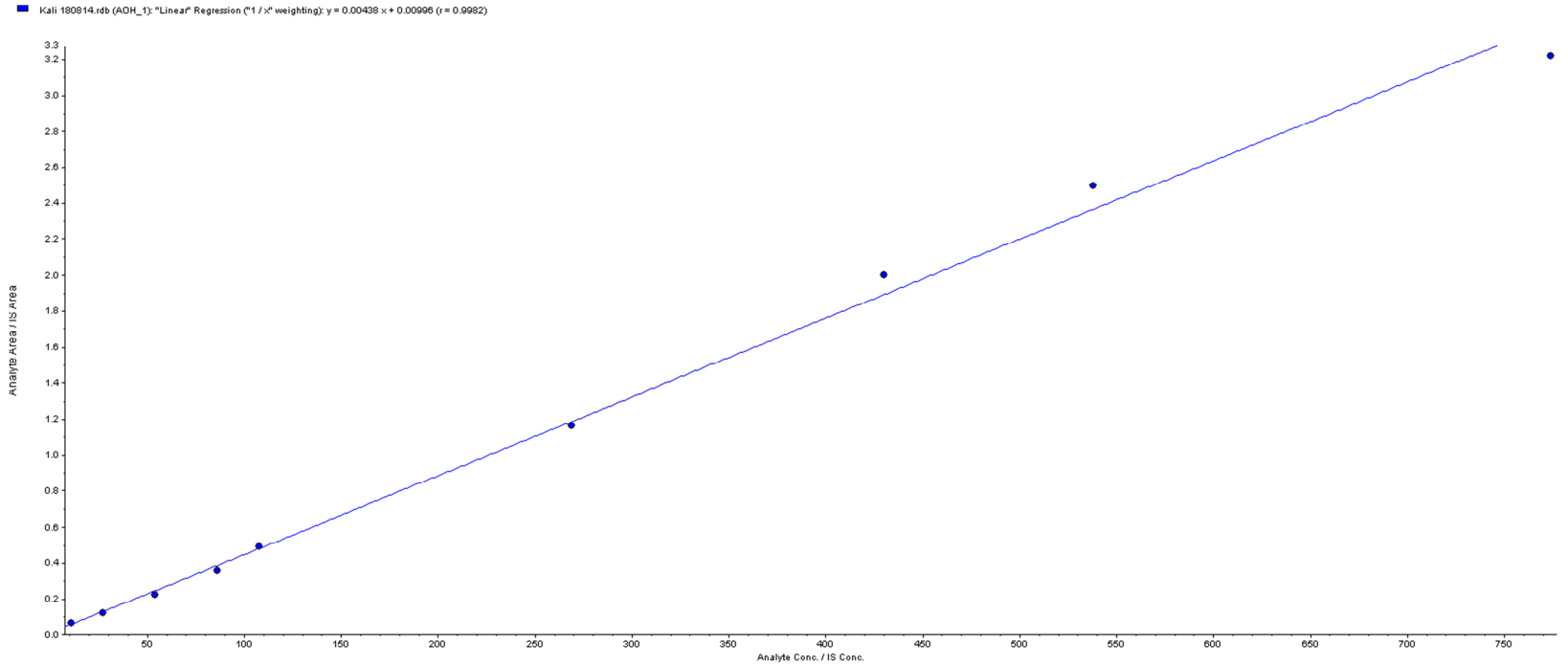


Fig. 2: Example for a calibration line for AOH (calibration ID 3 in **Tab. 6**) in mice blood (LC-MS/MS analysis)

Tab. 6: AOH – Calibration data; matrix: K3EDTA mice whole blood

		AOH									Regression parameters		
		Nominal concentration [ng / mL]									y=a*x+b; 1/x weighting		
Date	**Matrix**	**10.8**	**26.9**	**53.8**	**86.0**	**108**	**269**	**430**	**538**	**774**	**Intercept**	**Slope**	**R^2**
ID: 1													
30. Jul 14	mice	11.3	26.3	48.2	86.7	106	300	408	560	748			
Accuracy [%]	blood	*105*	*97.8*	*89.7*	*101*	*98.6*	*112*	*94.9*	*104*	*96.6*	*0.00898*	*0.00462*	*0.998*
ID: 2													
14. Aug 14	mice	12.8	25.2	53.2	79.9	99.9	266	441	681	780			
Accuracy [%]	blood	*119*	*93.8*	*99.0*	*92.9*	*92.9*	*99.0*	*103*	*127**	*101*	*0.00295*	*0.00461*	*0.999*
ID: 3													
18. Aug 14	mice	12.3	25.8	48.5	79.2	111	263	454	568	733			
Accuracy [%]	blood	*114*	*96.0*	*90.2*	*92.1*	*103*	*97.9*	*106*	*106*	*94.7*	*0.00996*	*0.00438*	*0.998*

** Accuracy > 115 %; does not comply with the EMA-guideline acceptance criteria; standard was not included in regression*

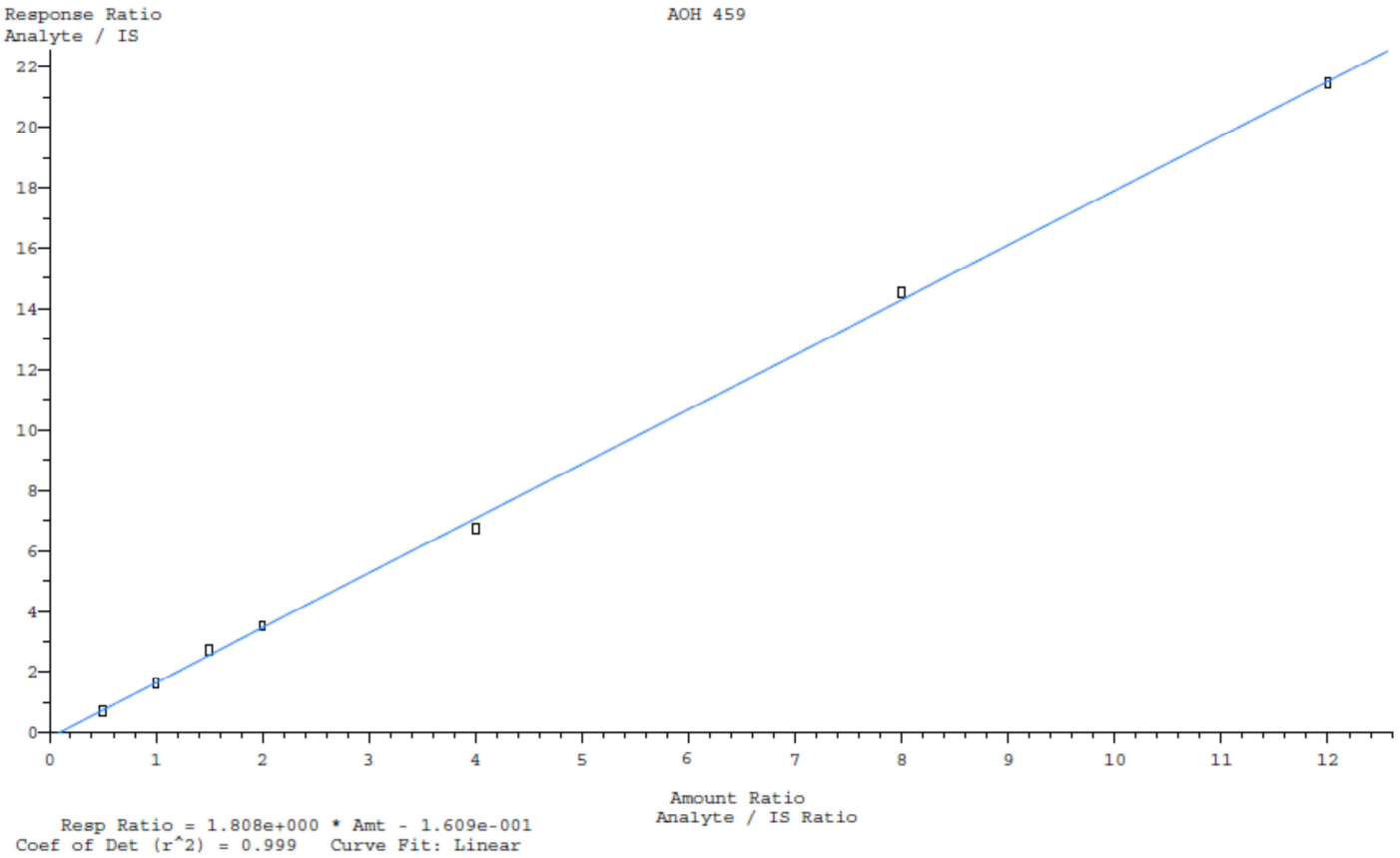


Fig. 3: Example for a calibration line for AOH (calibration ID 6 in **Tab. 7**) in mice urine (GC-MS analysis)

Tab. 7: AOH – Calibration data; matrix: mice urine

		AOH								Regression parameters		
		Nominal concentration [ng / mL]								y=a*x+b; no weighting		
Date	**Matrix**	**500**	**750**	**1000**	**2000**	**4000**	**6000**			**Intercept**	**Slope**	**R²**
ID: 1												
15. Aug 14	mice urine	111	657	951	2540	vial	5667	–	–			
Accuracy [%]	m/z 459	*22.3*	*87.5*	*95.1*	*127*	damaged	*94.5*	–	–			
										0.905	*1.660*	*0.982*
15. Aug 14	mice urine	253	742	1155	2335	vial	6089	–	–			
Accuracy [%]	m/z 459	*50.5*	*99.0*	*116*	*117*	damaged	*101*	–	–			
		500	**750**	**1000**	**2000**	**4000**	**6000**					
ID: 2												
15. Aug 14	mice urine	462	760	932	2053	vial	5996	–	–			
Accuracy [%]	m/z 222	*92.3*	*101*	*93.2*	*103*	damaged	99.9	–	–			
										-0.171	*0.334*	*0.999*
15. Aug 14	mice urine	536	808	1070	1859	vial	6025	–	–			
Accuracy [%]	m/z 222	*107*	*108*	*107*	*92.9*	damaged	*100*	–	–			

Tab. 7 (continued): AOH – Calibration data; matrix: mice urine

		AOH						Regression parameters		
		Nominal concentration [ng / mL]						y=a∗x+b; no weighting		
Date	**Matrix**	**50.0**	**125**	**250**	**375**			**Intercept**	**Slope**	**R²**
ID: 3										
15. Aug 14	mice urine	49.6	113	249	392	–	–			
Accuracy [%]	m/z 459	*99.3*	*90.2*	*99.7*	*104*	–	–			
								-0.0821	*2.50*	*0.992*
15. Aug 14	mice urine	52.6	130	260	355	–	–			
Accuracy [%]	m/z 459	*105*	*104*	*104*	*94.6*	–	–			
		50.0	**125**	**250**	**375**					
ID: 4										
15. Aug 14	mice urine	52.8	118	245	400	–	–			
Accuracy [%]	m/z 222	*106*	*94.5*	*97.8*	*107*	–	–			
								-0.00885	*0.266*	*0.989*
15. Aug 14	mice urine	53.8	129	255	356	–	–			
Accuracy [%]	m/z 222	*108*	*103*	*102*	*94.8*	–	–			
		500	**750**	**1000**	**2000**	**4000**	**6000**			
ID: 5										
15. Aug 14	mice urine	111	657	951	2540	vial	5667	–	–	
Accuracy [%]	m/z 459	*22.3*	*87.5*	*95.1*	*127*	damaged	*94.5*	–	–	
								0.9050	*1.66*	*0.982*
15. Aug 14	Urin	253	742	1155	2335	vial	6089	–	–	
Accuracy [%]	m/z 459	*50.5*	*99.0*	*116*	*117*	damaged	*101*	–	–	

Tab. 7 (continued): AOH – Calibration data; matrix: mice urine

		AOH								Regression parameters		
		Nominal concentration [ng / mL]								y=a*x+b; no weighting		
Datum	**Matrix**	**250**	**500**	**750**	**1000**	**2000**	**4000**	**6000**		**Intercept**	**Slope**	**R^2**
ID: 6												
20. Aug 14	mice urine	237	499	799	1015	1907	4062	5982	-			
Accuracy [%]	m/z 459	*95.0*	*99.7*	*106*	*101*	*95.3*	*102*	*99.7*	-	*-0.1610*	*1.81*	*0.999*
		50	**100**	**150**	**200**	**250**						
ID: 7												
22. Aug 14	mice urine	50.8	101	146	201	251	-	-	-			
Accuracy [%]	m/z 459	*102*	*101*	*97.2*	*101*	*100*	-	-	-	*-0.0754*	*2.04*	*0.999*

4.4 Carry Over - AOH

4.4.1 LC-MS/MS - mice whole blood

The carry over was assessed by injecting blank samples after QCH standards. The EMA guideline [1] stipulates that the signal response should be less than 20 % of the LLOQ for the analyte (AOH) and 5 % for the IS (HPC).

The selectivity data showed that the matrix contribution the AOH signal is subject of some variation, hence prior to starting the carry over experiment a blank sample (DoBl 300714-02) was injected. For the analyte AOH all blank samples, including DoBl 300714-02, have signal responses above 20 % of the LLOQ (**Tab. 8**). In order to account for the initial high AOH background corrected analyte peak areas were calculated (**Eq. 1**).

$$\textit{Analyte peak area (corrected)} = \textit{Reference DoBl peak area} - \textit{DoBl peak area} \qquad \textbf{(Eq. 1)}$$

The resulting signal responses in the blank samples varied between 0 and 17.6 % (**Tab. 8**), which is in agreement with EMA guideline criteria[1].

Tab. 8: Carry over for the analyte AOH and IS HPC

Sample Name	Sample Type	calculated conc [ng / mL]	Analyte Peak Area [counts]	IS Peak Area [counts]	Response % for AOH	Response % for IS
	LLOQ	11.8 (C.V.:5.99%)	29575 (C.V.:5.44%)	466250 (C.V.:7.54%)	-	-
DoBl 300714-02*	**Blank**	**-**	**11200**	**0**	**37.9**	**0**
QC 300714-17	QCH	496	1200000	523000	-	-
DoBl 300714-03	Blank	-	11100	0	37.5	0
QC 300714-18	QCH	513	1060000	447000	-	-
DoBl 300714-04	Blank	-	16400	0	55.5	0
QC 300714-19	QCH	482	1040000	467000	-	-
DoBl 300714-05	Blank	-	11300	0	38.2	0

* Blank sample ran before starting the carry over experiment.

corrected blank values						
	LLOQ	11.8 (C.V.:5.99%)	29575 (C.V.:5.44%)	466250 (C.V.:7.54%)	-	-
DoBl 300714-02#	**Blank**	**-**	**11200**	**0**	**37.9**	**0**
QC 300714-17	QCH	496	1200000	523000	-	-
DoBl 300714-03	Blank	-	-100	0	-0.3	0
QC 300714-18	QCH	513	1060000	447000	-	-
DoBl 300714-04	Blank	-	5200	0	17.6	0
QC 300714-19	QCH	482	1040000	467000	-	-
DoBl 300714-05	Blank	-	100	0	0.3	0

set as reference value

4.4.2 GC-MS analysis - mice urine

The carry over was assessed by injecting blank samples after QCH standards. The EMA guideline [1] stipulates that the signal response should be less than 20 % of the LLOQ for the analyte (AOH) and 5 % for the IS (DHC).

The consecutive injection of two blank samples after analysis of a QCH standard showed a pronounced carry over for the analyte AOH in the first blank samples (**Tab. 9**). There the AOH signal response varied between 5.57 and 11.62 %. The AOH signal response in the respective second blank samples was significantly lower with values varying between 0.22 and 0.27 %. Despite the observed carry over the obtained data demonstrate the compliance of this analytical method with the EMA guideline criteria [1].

For the internal standard the observed carry over was lower than for AOH (**Tab. 9**). And even though the difference between the consecutive blank samples was not as pronounced as for AOH, the data show a slightly higher signal response in the blank samples analysed immediately after the QCH standards. As for the analyte AOH the obtained data demonstrate the compliance of this analytical method with the EMA guideline criteria [1].

Tab. 9: Carry over for the analyte AOH and DHC

Sample Name	Sample Type	calculated conc [ng / mL]	Analyte Peak Area [counts]	IS Peak Area [counts]	Response % for AOH	Response % for IS
SG140805	LLOQ	249	4351040	3730977	-	-
SG140824	QCH	4997	56758964	*3278101*	-	-
SG140825	Blank		242350	56932	5.57	1.53
SG140826	Blank		9784	46268	0.22	1.24
SG140827	QCH	6172	69299858	3274934	-	-
SG140828	Blank		376542	60839	8.65	1.63
SG140829	Blank		10247	44087	0.24	1.18
SG140830	QCH	5411	58725355	3106436	-	-
SG140831	Blank		505413	63823	11.62	1.71
SG140832	Blank		11600	46408	0.27	1.24

4.5 Precision and Accuracy

4.5.1 LC-MS/MS - mice whole blood

Precision and accuracy of the analytical assay was assessed on LLOQ, QCL, QCM and QCH standards. The mean concentration for QCL, QCM and QCH standards was in all cases within 15 % (**Tab. 10**) of the respective nominal values as stipulated by the EMA guideline [1].

The mean concentration for the LLOQ standards was with 95.1 % (14. Aug 14) within 20 % of the nominal value as stipulated by the EMA guideline [1]. For the LLOQ standard (replicate injection of the same standard) analysed on 18. Aug 14 a mean accuracy of 79.5 % was calculated (**Tab. 10**), which was just outside the 80 % stipulated by the EMA guideline[1]. As this value can be rounded to 80 %, the value was considered as compliant.

The C.V. values did not exceed 15 % (**Tab. 10**) and hence, the determined precision and accuracy data for AOH comply with the EMA-guideline EMEA/CHMP/EWP/ 192217/2009.

Tab. 10: Precision and accuracy data –LC-MS/MS analysis

		AOH							
		LLOQ [ng / mL]	Accuracy [%]	QCL [ng / mL]	Accuracy [%]	QCM [ng / mL]	Accuracy [%]	QCH [ng / mL]	Accuracy [%]
Date	Calibration ID			25.0		100		500	
30. Jul 14	1	-		25.6	102	106	106	496	99.2
		-		28.7	115	111	111	513	103
		-		28.1	112	106	106	482	96.4
		-		29.1	116	106	106	500	100
		-		27.6	110	105	105	504	101
Mean (n = x)		-	n = 5	27.8	n = 5	107	n = 5	499	
SD (+/-)		-		1.22		2.14		10.2	
C.V. [%]		-		4.4		2.0		2.0	
Accuracy [%]		-		111		107		100	

Tab. 10 (continued): Precision and accuracy data – LC-MS/MS data

		AOH							
		LLOQ [ng / mL]	**Accuracy [%]**	**QCL [ng / mL]**	**Accuracy [%]**	**QCM [ng / mL]**	**Accuracy [%]**	**QCH [ng / mL]**	**Accuracy [%]**
Nominal value [ng / mL]		**10.8**		**35.8**		**86.0**		**430**	
14. Aug 14	**2**	9.22	85.4	31.9	89.1	82.0	95.3	433	101
		11.1	103	34.2	95.5	85.4	99.3	420	97.7
		10.5	97.2	33.3	93.0	84.5	98.3	420	97.7
		–		–		–		–	
		–		–		–		–	
Mean (n = x)	n = 3	10.3	n = 3	33.1	n = 3	84.0	n = 3	424	
SD (+/-)		0.784		0.946		1.438		6.128	
C.V. [%]		7.6		2.9		1.7		1.4	
Accuracy [%]		95.1		92.6		97.6		98.7	
Nominal value [ng / mL]		**10.8**		**35.8**		**86.0**		**430**	
18. Aug 14	**3**	7.78	72.0	34.1	95.3	92.6	108	485	113
		10.1	93.5	34.3	95.8	87.5	102	477	111
		8.12	75.2	–		–		–	
		8.76	81.1	–		–		–	
		8.80	81.5	–		–		–	
		7.97	73.8	–		–		–	
Mean (n = x)	n = 6	8.6	n = 2	34.2	n = 2	90.1	n = 2	481	
SD (+/-)		0.776		NA		NA		NA	
C.V. [%]		9.0		NA		NA		NA	
Accuracy [%]		79.5		95.5		105		112	

4.5.2 GC-MS - mice urine

Precision and accuracy of the analytical assay was assessed on QCL, QCM and QCH standards. The mean concentration for QCL, QCM and QCH standards was in all cases within 15 % (**Tab. 11**) of the respective nominal values as stipulated by the EMA guideline [1].

The C.V. values did not exceed 15 % (**Tab. 11**) and hence, the determined precision and accuracy data for AOH comply with the EMA-guideline EMEA/CHMP/EWP/192217/2009.

Tab. 11: Precision and accuracy data – GC-MS data

		AOH					
		QCL [ng / mL]	Accuracy [%]	QCM [ng / mL]	Accuracy [%]	QCH [ng / mL]	Accuracy [%]
Date	**Calibration ID**	**500**		**1000**		**4000**	
20. Aug 14	**6**	520	104	1052	105	4543	114
		538	108	1096	110	4434	111
		486	97.3	1087	109	4183	105
		528	106	1086	109	4562	114
		529	106	1108	111	4571	114
Mean (n = x)	n = 5	520	n = 5	1086	n = 5	4459	
SD (+/-)		17.9		18.6		146	
C.V. [%]		3.44		1.72		3.28	
Accuracy [%]		104		109		111	
20. Aug 14	**6**	499	100	1015	101	4062	102
		521	104	1027	103	4567	114
Mean (n = x)	n = 2	510	n = 2	1021	n = 2	4314	
SD (+/-)		NA		NA		NA	
C.V. [%]		NA		NA		NA	
Accuracy [%]		102		102		108	
21. Aug 14	**6**	488	97.7	902	90.2	3739	93.5
		452	90.3	1009	101	4161	104
Mean (n = x)	n = 2	470	n = 2	955	n = 2	3950	
SD (+/-)		NA		NA		NA	
C.V. [%]		NA		NA		NA	
Accuracy [%]		94.0		95.5		98.7	

Tab. 11 (continued): Precision and accuracy data – GC-MS data

		AOH					
		QCL [ng / mL]	Accuracy [%]	QCM [ng / mL]	Accuracy [%]	QCH [ng / mL]	Accuracy [%]
Date	**Calibration ID**	**500**		**1000**		**4000**	
22. Aug 14	**6**	504	101	1068	107	4403	110
		523	105	1118	112	4161	104
Mean (n = x)	n = 2	513	n = 2	1093	n = 2	4282	
SD (+/-)		NA		NA		NA	
C.V. [%]		NA		NA		NA	
Accuracy [%]		103		109		107	
Between run							
Mean (n = x)	n = 4	503	n = 4	1039	n = 4	4251	
SD (+/-)		19.7		55.8		186	
C.V. [%]		3.92		5.37		4.38	
Accuracy [%]		101		104		106	

4.5.3 Stability of AOH working solutions

The AOH working solutions were used for LC-MS/MS as well as GC-MS analysis. The stability of those solutions, when stored at approx. 4 - 8 °C (fridge) was assessed by LC-MS/MS only.

QCL and QCH standards were prepared from working solutions stored at approx. 4 – 8 °C (fridge) over a time period of 30 days. They were then quantified against a calibration line derived from freshly prepared working solutions.

The EMA-guideline [1] stipulates that QC standards should have accuracy values between 85 and 115 % of their nominal values. The mean accuracy values for the five replicates (**Tab. 12**) were 111 % for the QCL and 100 % for the QCH standards. Hence, it was concluded that AOH working solutions can be stored up to 30 days at approx. 4 – 8 °C (fridge).

Tab. 12: Stability of AOH working solutions stored at approx. 4 – 8 °C (fridge).

		AOH			
		QCL [ng / mL]	Accuracy [%]	QCH [ng / mL]	Accuracy [%]
Date	**Calibration ID**	**25.0**		**500**	
30. Jul 14	**1**	25.6	102	496	99.2
		28.7	115	513	103
		28.1	112	482	96.4
		29.1	116	500	100
		27.6	110	504	101
Mean (n = x)	n = 5	27.8	n = 5	499	
SD (+/-)		1.67		9.93	
C.V. [%]		6.0		2.0	
Accuracy [%]		111		100	

4.6 Autosampler stability

4.6.1 LC-MS/MS - mice whole blood

The autosampler stability of AOH in mice whole blood extracts was investigated under controlled (7 °C; **Tab. 13**) and uncontrolled conditions (no set temperature, **Tab. 14**) using QCL and QCH standards. Analyte stability could be demonstrated up to 30 h when stored in mice whole blood extracts at 7 °C (**Tab. 13**). The mean accuracy values for the QCL and QCH standards were within 15 % of their nominal values as stipulated by the EMA guideline[1].

Analyte stability in mice whole blood extracts at uncontrolled temperature could be established over a time period of 27 h (**Tab. 14**). The mean accuracy values for the QCL and QCH standards were within 15 % of their nominal values as stipulated by the EMA guideline[1].

Tab. 13: AS stability of AOH at 7 °C

	Nominal Conc. [ng / mL]	Calculated Conc. [ng /mL]	Accuracy [%]
Day 0 - QCL	25	25.6	102
	25	28.7	115
	25	28.1	112
	Mean	27.5	110
	SD (+ / -)	1.34	5.37
	C.V. [%]	4.9	4.9
Day 0 - QCH	500	496	99.2
	500	513	103
	500	482	96.4
	Mean	497	99.4
	SD (+ / -)	12.7	2.54
	C.V. [%]	2.6	2.6
Day x: up to 32 h[a] - QCL	25	27.1	108
	25	28.6	114
	25	29.0	116
	Mean	28.2	113
	SD (+ / -)	0.82	3.27
	C.V. [%]	2.9	2.9
Day x: up to 30 h[b] - QCH	500	497	99.4
	500	498	99.6
	500	Error*	NA
	Mean	498	99.5
	SD (+ / -)	NA	NA
	C.V. [%]	NA	NA

* sample not injected

[a] - time between the first QC standard injected on day 0 and the last standard injected on day x

[b] - time between the first QC standard injected on day 0 and the last standard injected on day x

Tab. 14: AS stability of AOH at uncontrolled temperature

	Nominal Conc. [ng / mL]	Calculated Conc. [ng /mL]	Accuracy [%]
Day 0 - QCL	35.8	34.1	95.3
	35.8	34.3	95.8
	35.8	31.9	89.1
	Mean	33.4	93.4
	SD (+ / -)	1.09	3.04
	C.V. [%]	3.3	3.3
Day 0 - QCH	538	485	90.1
	538	477	88.7
	430	433	101
	Mean	465	93.2
	SD (+ / -)	22.9	5.36
	C.V. [%]	4.9	5.8
Day x: up to 27 h[a] - QCL	35.8	35.4	98.9
	35.8	34.2	95.5
	35.8	33.3	93.0
	Mean	34.3	95.8
	SD (+ / -)	0.86	2.4
	C.V. [%]	2.5	2.5
Day x: up to 27 h[a] - QCH	538	487	90.5
	538	473	87.9
	430	420	97.7
	Mean	480	89.2
	SD (+ / -)	28.9	4.12
	C.V. [%]	6.0	4.6

* sample not injected

[a] - time between the first QC standard injected on day 0 and the last standard injected on day x

4.6.2 GC-MS - mice urine

The determination of AOH required its derivatisation with BSTFA over night. All samples and standards produced that way were analysed within a day. The analysis of study samples will be performed in such a way that the extracted samples will be bracketed with QC standards. This will allow to monitor the analyte integrity in the extracts during their autosampler storage prior to GC-MS analysis. The investigation of the autosampler stability during method validation was therefore omitted.

5 AOH metabolites

The validation parameters investigated for alternariol could not be covered for its four hydroxyl metabolites (8-hydroxy-AOH; 4-hydroxy-AOH; 10-hydroxy-AOH and 2-hydroxy-AOH) due to a lack of standards. However, in order to monitor those metabolites in mice urine as well as in mice whole blood by GC-MS analysis, alternariol was incubated with rat derived liver microsomes [2]. Microsomes contain cytochrome P450 enzymes which play a crucial role in the oxidative metabolism of xenobiotics. Hence, microsomal incubation of AOH results in the formation of its four hydroxyl metabolites (**Fig. 1**; **chapter 2**). Alternariol and its four hydroxyl metabolites were extracted by liquid-liquid extraction using ethyl acetate [2].
The peak assignment of the individual metabolites in the respective GC-MS chromatograms (**Fig. 2** and **3**) was confirmed in the associated Fraunhofer ITEM study applying NMR spectroscopy and mass spectrometry.

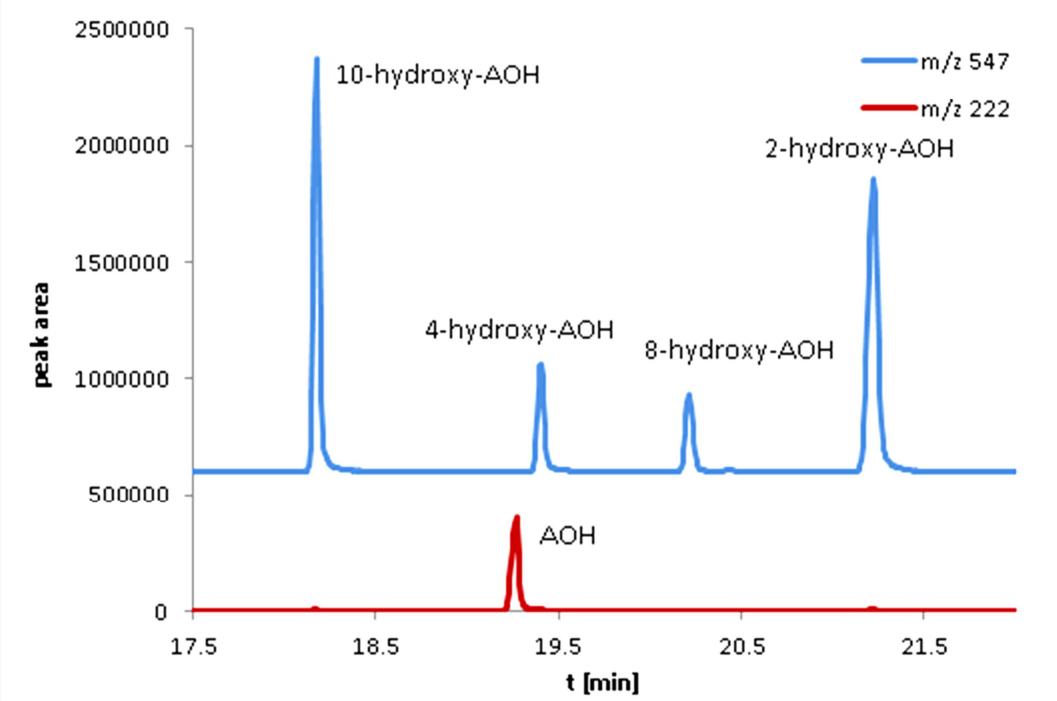


Fig. 2: AOH and its four hydroxyl metabolites in rat microsome extract. AOH monitored on m/z 222. Metabolites monitored on 547

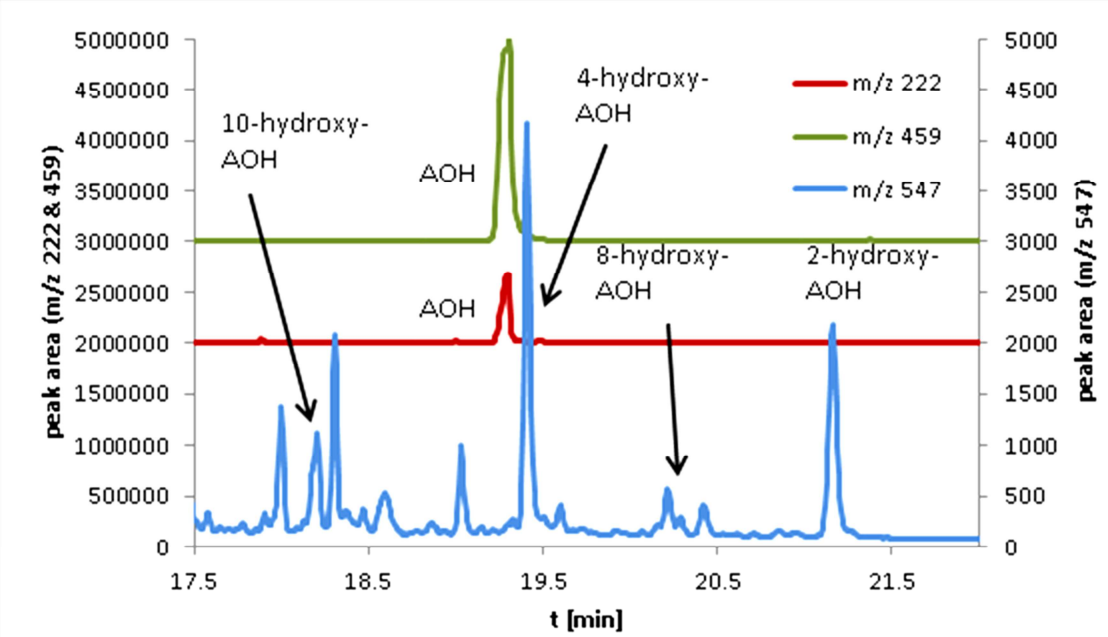


Fig. 3: Extracted urine sample. AOH monitored on m/z 222 and 459. Metabolites monitored on m/z 547

6 References

[1] EMA Guideline: EMEA/CHMP/EWP/192217/2009

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