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Metabolism of Methylisoeugenol in Liver Microsomes of Human, Rat and Bovine Origin.

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Running title: Metabolism of methylisoeugenol in liver microsomes

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Abbreviations: ARLM, Aroclor1254 pretreatment rat liver microsomes; BLM, bovine liver microsomes; CYP450, Cytochromes P450; DAD, diode array detector; FDA, Food and Drug Administration; FEMA, Flavor and Extract Manufacture Association; GRAS, 'Generally Recognized As Safe'; HLM, human liver microsomes, LC-MS; Liquid chromatography-mass spectrometry; NADP(H), Nicotinamide adenine dinucleotide phosphate; NMR, Nuclear magnetic resonance; RLM, rat liver microsomes; TLC, Thin layer chromatography

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Abstract

Methylisoeugenol (1,2-dimethoxy-4-propenylbenzene, **1**) is a minor constituent of essential oils, naturally occurring as a mixture of cis/trans isomers. **1** is an FDA approved food additive and has been given GRAS status. Previously, metabolism of **1** has been studied in the rat, revealing mainly non-toxic cinnamoyl derivatives as major metabolites. However, data concerning the possible formation of reactive intermediary metabolites are not available up to date. In this study, the oxidative metabolism of **1** was studied using liver microsomes of rat (not induced: RLM, Aroclor1254 induced: ARLM), bovine (BLM) and human (HLM, pooled from 150 donors) origin. Incubations of these microsomes with **1** provided phase-I metabolites that were separated by HPLC and identified by NMR-, UV/Vis-spectroscopy, and/or LC-MS-spectrometry. Identity was confirmed by comparison with ^{1}H-NMR spectra of synthesized reference compounds. Formation of metabolites was quantified by HPLC/UV using dihydromethyleugenol (**10**) synthesized as internal standard. From incubations of ARLM with **1**, seven metabolites could be detected, 3'-hydroxymethylisoeugenol (**2**), isoeugenol and isochavibetol (**3** + **4**), and 6-hydroxymethylisoeugenol (**5**) being the main metabolites. Secondary metabolites derived from **1** were identified as the α,β-unsaturated aldehyde 3'-oxomethylisoeugenol (**6**) and 1',2'-dihydroxy-dihydromethylisoeugenol (**7**). Surprisingly, formation of allylic 6-hydroxymethyleugenol (**8**) was observed starting about 30 min after beginning of incubations with ARLM. HLM did not form ring-hydroxylated metabolites, but were most active in the formation of **6** and **7**. ARLM incubations displayed the highest turnover rate and broadest metabolic pattern, presumably resulting from an increased expression of CYP450 enzymes. In conclusion, we present a virtually complete pattern of non-conjugated microsomal metabolites of **1** comprising reactive metabolites and suggesting the formation of reactive intermediates which need more investigation with respect to their possible adverse properties.

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Introduction

Methylisoeugenol (1,2-dimethoxy-4-propenylbenzene; **1,** see Fig. 5) is an alkenylbenzene naturally occurring as a mixture of cis/trans isomers in essential oils of *Asarum arifolium*, *Cymbopogon javanensis* and in nearly 60 other essential oils (Opdyke, 1975). It is used in perfumery as ingredient having a clove-carnation odor, and is applied as an additive to foods in the ppm range.

1 has been classified as 'Generally Recognized As Safe' (GRAS) by the Flavor and Extract Manufacture Association (FEMA) in 1965 and is approved by the FDA for food use (21CFR172.515). The acute oral LD_{50} in rats was reported to be 1.5 g/kg body weight (bw) and 2.5 g/kg bw (Opdyke, 1975). Adverse effects have not been seen in a feeding study with rats receiving 300 mg/kg bw per day (Purchase, 1992). **1** has overall been claimed not to be genotoxic (Hasheminejad, 1994), although definite data were missing.

Applied at full strength, **1** was irritating to skin (rabbit), but concentrations up to 8% in petrolatum did not produce irritations on human subjects. Using an equal concentration, **1** did not cause sensitization in a maximization test on human volunteers (Opdyke, 1975). However, sensitization was positive in a LLNA (local lymph node assay) on mice (Bertram, 1997).

The major metabolic pathway of **1** in the rat was found to be the oxidative transformation of the propenyl side chain leading to hydroxy and/or methoxy substituted cinnamic and benzoic acids. Other reactions were O-demethylation, and oxidation to 3,4-dimethoxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetone. Epoxidation of the side chain appeared to be a minor metabolic reaction (Solheim & Scheline, 1976). The metabolites reported in this previous study suggest the formation of reactive intermediates that may lead to hapten formation or may trigger other adverse effects.

In our study we intended to investigate the complete spectrum of metabolites of **1** formed by incubations with liver microsomes of different species.

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Material and Methods

Chemicals. Acetone, $CDCl_3$ and DMSO were purchased from Merck (Darmstadt, Germany). β- NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma-Aldrich/Fluka (Taufenkirchen, Germany). Methanol was obtained in HPLC gradient grade from J.T. Baker (Griesheim, Germany). Aroclor1254 was originally purchased from Monsanto (St. Louis, MO, USA). Methylisoeugenol (**1**), isoeugenol (**3**), methyleugenol (**11**) and DMSO-d_6 were purchased from Sigma-Aldrich (Steinheim, Germany) at a purity of > 98 %. The purity of the used chemicals was checked by HPLC analysis and/or NMR spectroscopy. Metabolites of **1** were synthesized according to procedures described below. The nomenclature of compounds and metabolites is following the numbering which is indicated in Fig. 5 for Methylisoeugenol (**1**).

Analytical Methods.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker DPX 400 or Avance 600 spectrometer in DMSO-d_6 (^{1}H-NMR: δ 2.49 ppm, ^{13}C-NMR: δ 39.5 ppm) or $CDCl_3$ (^{1}H-NMR: δ 7.26 ppm, ^{13}C-NMR: δ 77.0 ppm) at 298 K. TLC was performed on silica gel 60 F-254 plates (Merck, Darmstadt, Germany). IR spectra were recorded on a JASCO FT/IR-6100 spectrophotometer. The LC-MS/MS system consisted of a Perkin-Elmer LC-system coupled to an API 2000 Q trap instrument (Applied Biosystems). The mass spectrometer was operated using an Atmospheric Pressure Chemical Ionization source in the positive ion mode (APCI+). Acquisition and data processing were done with the Analyst software version 1.4 (Applied Biosystems). The instrumental settings used for the MS/MS analysis were as follows: ion source temperature 490 °C; declustering potential 19 V; needle current 5 V; focusing potential 364 V; entrance potential 8.5 V; curtain gas 19; ion source gas, 48; and turbo gas, 52, (the latter three are given in arbitrary units from the Analyst software). Analytical HPLC was carried out using an Agilent 1100 HPLC system equipped with an autosampler (G1329A), a

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quadruple pumping system (G1311A), a diode array spectrophotometer (DAD G1315A) and a column heater (G1316A; Agilent, Waldbronn, Germany). HPLC and LC-MS/MS separations were performed using a C18 column (Highbar®, 5 μm, 4.0 mm × 125 mm, LiChrosorb®, Merck, Darmstadt, Germany). The mobile phase system consisted of A (10 mM NH_4OAc in water), and B (pure MeOH). Separation was achieved using a 30-min linear gradient to 70% B from initial conditions of 10% B, following a 5-min period with constant eluent consistency of 70% B with a total analysis time of 38 min per sample at a flow-rate of 1 ml/min. The column temperature was kept constant at 35°C and detection wavelengths were 230, 261, and 280 nm with a reference wavelength of 340 nm each, and the detection wavelength of 340 nm with 450 nm as reference wavelength. Injection volumes were 50 μL each. Preparative HPLC was performed with an Agilent 1200 series system comprising two preparative pumps (G1361A), an automatic fraction collector (G1364B) and a multi-wave detector (G1315A). Injection was achieved over a manual valve with sample loops of 2.0 ml (incubations) or 10.0 ml (synthesis) volume. As solid phase, RP18 (Reprosil 100, 5 μm, 250 x 20 mm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was used.

Quantification

For quantification of the formed microsomal metabolites, the synthesized reference compounds were measured at concentrations of 1.0, 10, 50, 100, 500, and 1000 μM in methanol with the HPLC-gradient described above. Injection volumes were 50 μL each. Peak areas (AUC) detected at four different wavelengths (λ = 230, 261, 280 and 340 nm) were plotted versus concentration. Metabolite concentrations were calculated with the slope of the linear regression ($R^2 > 0.99$ for each compound) at the most sensitive wavelength.

Internal Standard

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As internal standard, dihydromethyleugenol (1,2-dimethoxy-4-propylbenzene, **10**) was synthesized according to the method given below. Its retention time was 31.0 min, a time when no metabolites were found to elute.

Microsomes

All animal experiments (rats) were performed according to National Animal Welfare Regulations after authorization by the local authorities (Struktur- und Genehmigungsbehörde Rheinland-Pfalz). Male Wistar rats were purchased from Charles River (Sulzbach, Germany). Animals were kept under a 12 h light/dark cycle and received water and commercial lab chow *ad libitum*. For inducing experiments, rats (body weight 180 to 250 g) were given a single dose of 500 mg/kg bw Aroclor1254 i.p. from a stock solution of 200 mg/ml Aroclor1254 dissolved in corn oil. Five days after treatment the animals were sacrificed. Aroclor1254-treated rat (ARLM), noninduced rat (RLM) and bovine liver microsomes (BLM) were prepared from the liver of freshly sacrificed/slaughtered animals as described for rat liver by Melancon et al. (1985). Bovine livers were from 1.5 -2 years old female German Black Pied Cattle (dairy cattle) and were obtained from a local slaughterhouse. Human liver microsomes (HLM) were purchased from BD Bioscience (Heidelberg, Germany), from a pool derived from 150 gender-mixed donors. Protein was measured photometrically by the method of Bradford (1976). Total cytochrome P450 contents were measured according to the method of Omura and Sato (1962).

Incubations

Incubations of microsomes (2.0 mg microsomal protein/ml) were performed with 0.1, 0.5 or 2.5 mM substrate dissolved in DMSO (final concentration 5%) with a NADPH-regenerating system consisting of 1.0 mM NADPH, 0.5 units glucose-6- phosphate dehydrogenase, 5.0 mM glucose-6-phosphate, 3.0 mM magnesium chloride, and 50 mM phosphate buffer, pH 7.4. Incubations were carried out in various volumes (from 1.0 ml up to 100 ml for

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preparative uses), gently shaked in an incubator (TH-30, Carl Roth, Karlsruhe, Germany) at 37°C. After pre-incubation with substrate for 5 min at 37°C, the NADPH-generating system was added and the mixture was incubated for up to 48 h. Control incubations were carried out with heat-inactivated microsomes and with intact microsomes but without NADPH-regenerating system, respectively. Incubations and time course measurements were done in triplicate as independent experiments. The high concentration of DMSO (5%) was applied to improve solubility of the substrate, in particular of isolated metabolites. Although DMSO is known to act as CYP inhibitor, concentrations of metabolites in control incubations of **1** using 0.5% DMSO were found not to be significantly different (data not shown).

Sample preparation

After incubation, samples were diluted with equal volumes of a cold solution (-20°C) of acetone containing 0.1 or 0.5 mM **10** as internal standard. Samples were cooled for 20 min on ice. The mixtures were shaken intensely and the precipitated protein was removed by centrifugation at 13,000 g for 5 min. For time course measurements, the supernatants were used directly for HPLC analysis. The supernatants of single incubations were extracted five times with an equal volume of ethyl acetate. The solvent of the combined organic phases was removed and the residue was taken up in a defined volume of methanol prior to further analysis.

Synthesis

3'-Hydroxymethylisoeugenol (**2**) was prepared according to the method of Solheim & Scheline (1976) using **9** (as starting material. Yield: 229 mg (1.18 mmol, 42 %, light yellow oil). Analysis: R_f = 0.36 (EtOAc/Hexan 1:1, v/v); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.72 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 4.07 (pt, 2H, H3'), 4.80 (t, 1H, OH, J = 5.4 Hz), 6.23 (dt, 1H, H2', J = 16.0 Hz, J = 5.4 Hz), 6.44 (d, 1H, H1', J = 16.0 Hz), 6.88 (ps; 2H, H5, H6), 7.03 (s,

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1H, H2); t_R (HPLC-UV) = 16.7 min (280 nm; 500 µM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 214 (780), 263 (580), 300 (220) nm; IR (neat): 3583, 3414, 2255, 2128, 1659, 1050, 1025, 1005, 825, 764, 666 cm^{-1}.

(*E*)-6-Hydroxymethylisoeugenol (**5**) was synthesized according to Shulgin (1965) by isomerization of **8**. Under argon atmosphere, a mixture of **8** (4.5 g, 23.2 mmol) and potassium hydroxide (11.2 g; 0.20 mol) in dried ethanol (5 ml) was stirred at 140-150°C for 7 days. Reaction control was accomplished by analytical HPLC. Additional KOH (11.2 g; 0.20 mol) was added after 48 and 96 h, each. Reaction was stopped after 7 d, as no further conversion was observed. 500 ml of water were added the mixture was acidified with dilute sulfuric acid and extracted with ethyl acetate (4 times, 150 ml each). The combined organic layers were dried ($MgSO_4$) and the solvent was evaporated in vacuo. The crude product (4.2 g) was purified by column chromatography using silica gel and ethyl acetate/hexane (1:2). Crystallization from ethyl acetate/pentane yielded the pure *trans*-isomer **5** (2.8, 14.4 mmol, 62%) as light yellow needles. Analysis: R_f = 0.41 (EtOAc/hexane 1:2); ; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 1.7 (dd, 3H, CH_3, J = 6.4 Hz, J = 1.2 Hz); 3.66 (s, 6H, 2 OCH_3); 6.04 (m, 1H, H2'); 6.41 (s, 1H, H5); 6.51 (dd, 1H, H1', J = 16.4 Hz, J = 1.4 Hz); 6.83 (s, 1H, H2); 9.05 (s; 1H, OH); ^{13}C{^{1}H}-NMR (DMSO-d_6, 25°C, 100 MHz) δ 18.6, 55.3; 56.2; 100.9; 110.0; 115.5; 121.9; 125.6; 142.0; 148.2; 148.6 ppm; IR (KBr): 3405, 3040, 3009, 2995, 2956, 2932, 2848, 1615, 1524, 1454, 1419, 1375, 1297, 1266, 1204, 1115, 1033, 999, 970, 940, 873, 827, 753, 684, 538, 472 cm^{-1}; t_R (HPLC-UV; 261 nm, 500 µM) = 21.5 min; UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 215 (1100), 257 (700), 315 (350) nm.

(*E*)-3'-Oxomethylisoeugenol (6) was synthesized according to the method of Bürgi et al. (1993) using **2** as starting material. Separation by preparative HPLC yielded 119 mg (0.62

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mmol, 47 %) of the pure *trans*-product as pale yellow crystals. Analysis: R_f = 0.39 (EtOAc/hexane 1:2); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3.80 (s, 6H, 2 OCH_3); 6.78 (dd, 1H, H2', J = 7:8 Hz, J = 15.6 Hz); 7.02 (d, 1H, H5, J = 8.2 Hz); 7.27 (dd, 1H, H6, J = 8.2 Hz, J = 1.6 Hz); 7.35 (d, 1H, H2, J = 1.6 Hz); 7.62 (d, 1H, H1', J = 15.6 Hz); 9.60 (d, 1H, H3', J = 7.8 Hz); IR (KBr): 3437, 2929, 2838, 1667, 1619, 1597, 1512, 1469, 1426, 1400, 1273, 1225, 1165, 1129,1037, 1017, 984, 794, 581 cm^{-1}; t_R (HPLC-UV) = 19.0 min (261 nm; 1.0 mM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 230 (2600), 240 (3000), 300 (3000), 360 (2750) nm.

1',2'-Dihydroxymethylisoeugenol (**7**). To a solution of **1** (1.00 g, 5.61 mmol) in dichloromethane (15 ml) was added a mixture (20 ml 1:1, v/v) of a solution of saturated sodium bicarbonate and a solution of saturated ammonium chloride. A solution of *meta*-chloroperbenzoic acid (70%, 1.45 g, 5.88 mmol) in dichloromethane (10 ml) was added dropwise. The mixture was vigorously stirred for 2 h at room temperature. The organic layer was separated and the aqueous layer was extracted with dichloromethane three times. The combined organic phases were washed three times with water, dried over $MgSO_4$ and the solvent was removed in vacuo to yield 1-(3,4-dimethoxyphenyl)-1-hydroxypropane-2-yl-3-chlorobenzoate (1.90 g 5.42 mmol; 97 %) as a white solid. Analysis: R_f = 0,31 (EtOAc/hexane 1:2); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 0.95 (d, 3H, CH_3); 3.71 (s, 3H, OCH_3); 3.74 (s, 3H, OCH_3); 4.05 (sext, 1H, H2'); 5,14 (d, 1H, H1'); 5.59 (d, 1H, OH); 6.90 (d, 1H, H5, J = 7.8 Hz); 6.94 (dd, 1H, H6, J = 7.8 Hz, J = 1.7 Hz); 7.00 (d, 1H, H2, J = 1.7 Hz); 7.56 (t, 1H, H5'', J = 7.8 Hz); 7.72 (dd, 1H, H4'', J = 8.2 Hz, J = 1.4 Hz); 7.98 (d, 1H, H6'', J = 7.8 Hz); 8.07 (t, 1H, H2'', J = 1.4 Hz); IR (KBr): 3515, 3069, 2972, 2936, 2908, 2837, 1721, 1606, 1594, 1518, 1465, 1422, 1346, 1321, 1256, 1161, 1139, 1087, 1073, 1027, 978, 897, 866, 849, 809, 793, 749, 737, 702, 674, 665, 645 cm^{-1}.

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This intermediate was used without further purification. To 1.83 g (5.23 mmol) of this intermediate in methanol (30 ml), sodium hydroxide (1.56 g, 39.0 mmol) in methanol (10 ml) was added. The solution was refluxed for 1 h till no starting material was detectable by TLC. After cooling to room temperature, the solution was acidified with 1 N HCl and adjusted to pH 10 using a saturated solution of ammonium bicarbonate. The aqueous layer was extracted three times with ethyl acetate (EtOAc; 30 ml). The combined organic layers were washed with brine and water, dried ($MgSO_4$) and the solvent was evaporated in vacuo. Yield: 1.00 g (4.74 mmol, 91 %, white solid). Analysis: R_f = 0.14 (EtOAc/hexane 1:3); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 0.81 (d, 3H, H3', J = 6.2 Hz); 3.61 (m, 1H, H2'); 4.18 (m, 1H, H1'); 4.51 (d, 1H, OH, J = 4.3 Hz); 5.05 (d, 1H, OH, J = 4.3 Hz); 6.79 (dd, 1H, H6; J = 8.2 Hz, J = 1.6 Hz); 6.85 (d, 1H, H5, J = 8.2 Hz); 6.88 (d, 1H, H2, J = 1.6 Hz); IR (KBr): 3411, 3274, 2964, 2930, 2834, 1606, 1594, 1520, 1460, 1420, 1373, 1343, 1260, 1239, 1160, 1146, 1079, 1057, 1025, 910, 819, 789, 764, 644, 618, 598, 543, 421 cm^{-1}; t_R (HPLC-UV) = 10.8 min (280 nm; 500 μM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 230 (135), 277 (35) nm.

6-Hydroxymethyleugenol (**8**) was synthesized according to Benbow et al., 2001 (Procedure B) using 3,4-dimethoxyphenol (4.0 g, 26.0 mmol) as starting material. Allylation of the phenolic hydroxyl group led to the required allylether (99%). Subsequent Claisen-rearrangement provided **8** (4.9 g, 25.2 mmol, 97%) as white crystals. Analysis of **8**: R_f = 0.43 (EtOAc/hexane 1:2); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3.71 (s, 3H, OCH_3); 3.72 (s, 3H, OCH_3); 4.97 (t, 1H, H3'Z, J = 5.1 Hz); 5.03 (d, 1H, H3'E, J = 10.2 Hz); 5.22 (dt, 1H, H1', J = 17.0 Hz); 5.41 (d, 1H, OH, J = 4.4 Hz); 5.93 (m; 1H, H2'); 6.82 (dd, 1H, H6, J = 8.5 Hz, J = 1.6 Hz); 6.87 (d, 1H, H5, J = 8.5 Hz); 6.90 (d, 1H, H2, J = 1.6 Hz); ^{13}C{^{1}H}-NMR (DMSO-d_6, 25°C, 100 MHz) δ 33.3, 55.5; 56.5; 101.0; 114.8; 114.9; 116.6; 137.6; 141.6; 147.8; 148.8 ppm; IR (neat): 3445, 3077, 3002, 2937, 2911, 2836, 1637, 1617, 1523, 1465, 1451, 1415, 1360, 1295, 1238, 1203, 1114, 1030, 999, 916, 847, 756, 666 cm^{-1}; t_R (HPLC-UV) = 20.0 min

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(280 nm; 500 µM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 230 (260), 290 (170) nm.

1'-Hydroxymethyleugenol (**9**) was prepared according to the method of Borchert (1973) using veratraldehyde as starting material. Yield: 2.25 g (11.6 mmol, 96%, light yellow oil). Analysis: R_f = 0.32 (EtOAc/hexane 1:2); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3.71 (s, 3H, OCH_3); 3.72 (s, 3H, OCH_3); 4.97 (t, 1H, H3'$_Z$, J = 5.1 Hz); 5.03 (d, 1H, H3'$_E$, J = 10.2 Hz); 5.22 (dt, 1H, H1', J = 17.0 Hz); 5.41 (d, 1H, OH, J = 4.4 Hz); 5.93 (m; 1H, H2'); 6.82 (dd, 1H, H6, J = 8.5, 1.6 Hz); 6.87 (d, 1H, H5, J = 8.5 Hz); 6.90 (d, 1H, H2, J = 1.6 Hz); ^{13}C{^{1}H}-NMR (DMSO-d_6, 25°C, 150 MHz) δ 55.9; 56.1; 73.7; 110.7; 112.2; 113.5; 118.8; 137.4; 142.7; 148.3; 149.1 ppm; IR (neat): 3504, 3583, 3504, 3078, 3002, 2936, 2836, 1735, 1640, 1594, 1515, 1464, 1418, 1374, 1339, 1262, 1234, 1186, 1155, 1139, 1028, 992, 926, 860, 813, 785, 763, 666 cm^{-1}; t_R (HPLC-UV) = 16.4 min (280 nm; 500 µM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 233 (280), 278 (100) nm.

Dihydromethyleugenol (**10**) In a hydrogenation apparatus, 289 mg of Pd/C (10%) were added to a solution of methyleugenol (**11**) (3.00 g; 16.8 mmol) in ethanol (30 ml). The suspension was stirred vigorously under slight excess pressure at RT until 381 cm³ of hydrogen (17.0 mmol) were taken up. The charcoal was filtered off and the solvent was removed in vacuo. Yield: 1.09 g (6.60 mmol, 99 %, colorless oil). Analysis: R_f = 0.57 (EtOAc/hexane 1:4); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 0.86 (t, 3H, H3', J = 7.5 Hz); 1.54 (sext, 2H, H2', J = 7.5 Hz); 2.46 (t, 2H, H1', J = 7.5 Hz); 3.69 (s, 3H, OCH_3); 3.71 (s, 3H, OCH_3); 6.66 (dd, 1H, H6, J = 8.2 Hz, J = 1.7 Hz); 6.75 (d, 1H, H2, J = 1.7 Hz); 6.81 (d, 1H, H5; J = 8.2 Hz); IR (KBr): 2997, 2957, 2932, 2870, 2834, 1607, 1590, 1516, 1465, 1416, 1377, 1341, 1262, 1236, 1191, 1156, 1141, 1092, 1031, 846, 806, 764, 666 cm^{-1}; t_R (HPLC-

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UV) = 31.0 min (280 nm; 500 μM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 230 (270), 280 (100) nm.

2',3'-Dihydroxymethyleugenol (**13**) was synthesized with 2',3'-epoxy-methyleugenol (101 mg; 0.52 mmol, prepared by the method of Fieser, 1967). The starting material was mixed with 5 ml of water at 25°C. Solid potassium hydroxide (270 mg, 15.1 mmol) was added and the mixture was refluxed for 2 h. After cooling to room temperature, 6N HCl was added up to pH 5-6. The mixture was extracted three times with ethyl acetate, the combined organic layers were dried ($MgSO_4$), filtered, and concentrated in vacuo to yield **13** as a white solid (76.0 mg, 0.36 mmol, 69%). Analysis: R_f = 0.19 (EtOAc/hexane 1:2); ^{1}H-NMR (DMSO-d_6, 600 MHz) δ 2.45 and 2.67 (m, 2H, H1'), 3.26 (m, 2H, H3'), 3.59 (m, 1H, H2'), 3.69 (s, 3H, OCH3), 3.71 (s, 3H, OCH3), 4.51 (ps, br, 2H, OH), 6.69 (dd, 1H, H6, J = 8.2 Hz, J = 1.8 Hz), 6.79 (d, 1H, H2, J = 1.8 Hz), 6.81 (d, 1H, H5, J = 8.2 Hz); ^{1}H-NMR ($CDCl_3$, 25°C, 400 MHz) δ 2.47 (m, 2H, H1'), 3.13 and 3.20 (m, 2H, H3'), 3.50 (s, 3H, OCH_3), 3.52 (s, 3H, OCH_3), 3.63 (m, 1H, H2'), 6.46 (m, 3H, ArH); t_R (HPLC-UV) = 10.5 min (280 nm, 1.0 mM); UV/Vis (HPLC-DAD): λ_{max} (absolute absorbance in mAU) = 230 (2100), 280 (850) nm;

(*E*)-3,4-Dimethoxycinnamic acid (14) was synthesized starting from ferulic acid (1,06 g; 5,46 mmol). After methylation (2.80 g, 20.3 mmol of K_2CO_3 and 2.78 g, 21.84 mmol, 2.0 ml of dimethyl sulfate) in acetone the crude product was dissolved in methanol and saponificated with KOH (2.58 g, 46.0 mmol) to yield 1.10g (5.30 mmol, 97 %) of pure *trans*-**14** as a white solid. Analysis: R_f = 0,24 (EtOAc/Hexan 1:2); ^{1}H-NMR (DMSO-d_6, 600 MHz, 295K) δ 3.78 (s, 3H, OCH_3); 3.79 (s, 3H, OCH_3); 6.42 (d, 1H, H2', J = 15.8 Hz); 6.96 (d, 1H, H5, J = 8.2 Hz); 7.18 (dd, 1H, H6, J = 8.2 Hz, J = 2.0 Hz); 7.30 (d, 1H, H2, J = 2.0 Hz); 7.50 (d, 1H, H1', J = 15.8 Hz); 12.19 (s, br, 1H, COOH); IR (KBr): 3447, 2937, 2841, 1683, 1625, 1596, 1584, 1516, 1457, 1426, 1408, 1340, 1315, 1299, 1263, 1210, 1169, 1141,

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1024, 976, 939, 840, 811, 580 cm^{-1}; t_R (HPLC-UV) = 6.5 min (280 nm; 0.5 mM); UV/Vis (HPLC-DAD): λ_{max} (absolute Absorption in mAU) = 215 (600), 230 (540), 285 (590), 312 (580) nm.

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Results

Human, bovine, and rat (Aroclor1254-induced and non-induced) liver microsomes were incubated with methylisoeugenol (**1**) in the presence of a NADPH generating system to generate metabolites formed by metabolic phase-I reactions. Incubations without NADPH generating system did not produce metabolites in detectable amounts (data not shown). Relatively high concentrations (100 µM and 500 µM) of **1** were used with incubations to ensure detection of lesser formed metabolites.

After workup, supernatants of the incubations were analyzed by RP-HPLC/DAD. Peaks of metabolites were separated and examined using HPLC/MS and ^{1}H-NMR spectroscopy. Formation of the main metabolites and decrease in substrate concentration were monitored in microsomal incubations of different species and different concentrations. The total content of CYP-enzymes in these incubations was determined to be 1.67 (ARLM), 0.33 (RLM), 0.88 (BLM), and 0.35 (HLM) nmol/mg protein.

Oxidative metabolism of methylisoeugenol (1)

Incubations of ARLM with **1** produced the widest spectrum of metabolites, **2 - 8** displaying the largest peak areas at a substrate concentration of 500 µM (Fig. 1). These metabolites were identified by comparison of HPLC retention times and UV spectra to those of synthetic reference compounds, if available.

The favored metabolic steps in ARLM were the hydroxylation of the propenyl side chain leading to 3'hydroymethyleugenol (**2**), the hydroxylation at position 6 of the aromatic ring leading to **5** and **8**. Furthermore, the 1',2'-diol **7** was identified. The products of demethylation of one of the methoxy groups were identified as **3** (isoeugenol) and characterized as **4** (isochavibetol). Although no reference compound was available for **4**, its formation in the incubation mixtures with liver microsomes was tentatively confirmed by analyzing its MS data ($[M+H]^+$ = 165 m/z). Furthermore, its UV-spectrum (215 (1200 units),

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260 (800 units), 300 (450 units) nm) was identical (data not shown) to the spectrum of isoeugenol (**3**), as expected. Interestingly, **5** could be detected already 10 min after NADPH addition while **8** was first detected 30 min after addition of the NADPH generating system. This metabolic transformation was found only in incubations with ARLM. However, the concentration of **5** decreased during further incubation, i.e., **5** could barely be detected after 24 h of incubation. After 2 h the formation of **6** became observable indicated by a 'negative' peak. This is due to the used reference wavelength (340 nm) where **6** exclusively shows a marked absorption. Quantification of this metabolite was achieved using a detection wavelength of 340 nm and a reference wavelength of 450 nm.

In Fig. 2A-D, the time-course of levels of metabolites formed during incubations of **2** (500 μM) with liver microsomes of different origins is shown. The remaining substrate methylisoeugenol (**1**) and the major metabolites **2-7** could be quantified. For clarity of the figures, the concentrations of the metabolites **3, 4, 6** and **7** are shown as sum. The fact, that the calculated sum of concentrations of substrate and quantified metabolites was less than 100%, points to the formation of further metabolites or reaction products, the amounts of which could not be specified. This difference was found to increase with progressive time of incubation suggesting the formation of conjugated metabolites and/or of metabolites binding to macromolecules. In ARLM (Fig, 2A), the strongest decline of **1** over time was observed. The 3'-alcohol **2,** the ring-hydroxylated products **5** and **8**, and the sum of the minor metabolites **3**, **4** and **7** almost equally contributed to the overall yield of metabolites. In contrast, the slightly lower yield of metabolites in microsomes from untreated rats (RLM) mainly consisted of **2** (Fig. 2B) with some minor formation of **3** + **4** and **6** but a lack of **5, 7** and **8**. In bovine liver microsomes (BLM), **2** also dominated while lower amounts of **5**, **6** and **7**, and of the sum of **3** + **4,** were found (Fig. 2C). In human liver microsomes (Fig. 2D), a similar picture was obtained with respect to the dominating role of **2.** Besides some formation of **3** + **4** and **7**, however no formation of **5** nor **8** could be detected.

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In Table 1, the amounts of metabolites formed in ARLM, RLM, BLM, and HLM is expressed as percentage of the substrate concentration of **1** (at 100 µM or 500 µM starting concentration, respectively) at the start of the incubation. At the lower substrate concentration of 100 µM, only levels after 2 h of incubation were monitored since a number of metabolites were below the limits of detection at earlier time points. In contrast, the metabolic capacity of ARLM was sufficient to lead to an almost complete loss of metabolites after 2 h. Therefore, Table 1 shows the pattern of metabolites for ARLM at 100 µM substrate concentration after 10 min only. At a substrate concentration of 100 µM, **5** became the dominating metabolite in ARLM, followed by **2** and the minor metabolites **3**, **4**, **7** and **8** . In RLM, the relative yield of **2** was also decreased at the lower substrate concentration, which was not the case, however, in BLM and HLM. In HLM, decrease in substrate concentration even seemed to slightly enhance the relative formation of **2** while formation of **7** was strongly enhanced.

Incubation of synthesized metabolites 3'-hydroxymethylisoeugenol (2) and 6-hydroxymethylisoeugenol (5) with liver microsomes

For the elucidation of the metabolic pathways, and for a more precise specification of minor metabolites generated during incubations with **1**, ARLM were incubated with compound **2**, i.e., 3'-hydroxy-methylisoeugenol, synthesized as reference substance. After work-up, the supernatants of these incubations were applied to HPLC analysis.

Five secondary metabolites were detected besides non-metabolized substrate (57%) after 3h (Fig. 3). The 'negative peak' eluting at 19.0 min was identified as the aldehyde **6**, resulting from microsomal oxidation of the hydroxy group in 3'-position. The compound with t_R = 10.5 min showed intense absorption at 280 nm (Fig. 3A), and a weaker absorption at 261 nm (Fig. 3B), more characteristic for an allyl or saturated side chain attached to the phenyl ring. The peak at t_R = 6.3 min displayed spectroscopic characteristics indicating an α,ß-unsaturated oxo compound. According to their retention times and spectral properties, both of these

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compounds are presumed to be 2',3'-dihydroxy-dihydromethyleugenol (**13,** t_R = 10.5 min), and 3,4-dimethoxycinnamic acid (**14**, t_R = 6.3 min). In contrast, the compounds with t_R = 11.7 and t_R = 12.3 min displayed strong absorbance at 261 nm and weaker absorbance at 280 nm, being characteristic for compounds carrying a 2-propenyl side chain. The retention times point to dihydroxy derivatives of **1,** e.g., bearing one hydroxyl group at the side chain and another at the aromatic ring. However, preparative separation of these secondary metabolites failed, and so identification via NMR-spectra was not feasible.

Since the formation of **8**, representing an allylic compound, during incubation of ARLM with **1** appears uncommon, we also investigated the metabolism of synthethic **5**. In Fig. 4, chromatograms of incubation supernatants of HLM with **5** (100 µM, 2h, t_R = 21.5 min) are depicted. The concentration of **8** (t_R = 20.0 min) continuously increased during incubation period. At a retention time of 16.3 min, a metabolite was formed displaying a 'negative peak'. The UV-spectrum of this compound showed a maximum of absorption at 340 nm, indicative for an α,ß-unsaturated oxo compound. The metabolic transformation of **5** leading to **8** was observed for all liver microsomes tested using 100 µM starting concentration of **5** with a ranking HLM > ARLM > BLM > RLM (data not shown). However, using higher starting concentrations of **5** (500 µM and 1.0 mM) the formation rate of **5** rapidly decreases (data not shown).

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Discussion

Whereas previous investigations on incubations of liver microsomes treated with methyleugenol (**11**) as a substrate (Jeurissen et al., 2006; Smith et al., 2002; Gardner et al., 1997) were performed to clarify the formation of the proximate carcinogen 1'-hydroxymethyleugenol (**9**), no reports dealing with investigations on the metabolism of methylisoeugenol (**1**) in liver microsomes are available.

Incubation with microsomes from untreated and Aroclor1254-treated rats, and of bovine and human origin revealed a more or less complex pattern of phase I metabolites depicted in Figure 5. As discussed in the following, some of the metabolites shown were not found in all incubations.

The main metabolite in nearly all types of incubations, the 3'-hydroxy metabolite **2**, is considered as a non-mutagenic product. However, our study revealed that it can be converted into the α,ß-unsaturated aldehyde **6** probably being capable of forming Michael adducts. Such reactions with, e.g., amino acids and proteins, possibly play a role in liver toxicity and hapten formation. **6** is likely to be metabolized further to the corresponding carboxylic acid **14**.

In contrast to incubations with methyleugenol, whereupon isomerization of the side chain and hydroxylation in 3'-position was found (unpublished data), the corresponding isomerization of **2** resulting in the formation of 1'-hydroxymethyleugenol **(9)** was not observed in our experiments. This is in agreement with previous investigations which showed a lack of genotoxicity of **1** in rat hepatocytes in culture (Hasheminejad & Caldwell 1994). Starting with a propen-1-yl side chain, the formation of an allylic cation obviously does not seem to be energetically advantageous. However, as early as 30 min after starting the ARLM incubation with **1**, the formation of **8** was observed suggesting that the hydroxy group adjacent to the propenyl side chain may facilitate isomerization to the corresponding allyl compound. Similar findings have been reported for the metabolism of asarones. These molecules, bearing a methoxy group instead of the hydroxyl group adjacent to the propenyl side chain, have been

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found to be genotoxic, although they cannot undergo 1'-hydroxylation directly, which is the key step in bioactivation and responsible for the genotoxic properties of many allyl compuonds such as methyleugenol, safrole or estragole (Hasheminejad & Caldwell 1994). Incubations of liver microsomes with synthethic **5** showed the capacity to isomerize the propenylic into an allylic side chain. However, it should be noted that the metabolic formation of **5** was observed only in incubations of ARLM or BLM.

Further metabolites were the mono-demethylated metabolites isoeugenol (**3)** and isochavibetol (**4**), and the 1',2'-diol **7**. The demethylated products **3** and **4** were found in all incubations with the highest amounts present in HLM and ARLM. Lower amounts were found in RLM and BLM.

Methylisoeugenol-1,2-epoxide (**12**) could not be detected. The formation of the diol **7,** however, most likely originates from the intermediary formation and further reaction of **12**. Metabolite **7** continuously increased and reached its highest levels after 24 h in ARLM, HLM, BLM, while it was below the limit of detection in non-induced RLM.

The total recovery of metabolites together with remaining starting material was above 70 % after the first 3 hours of incubation in all types of microsomes investigated. The turnover rate of **1** was highest in ARLM, and lower in HLM, BLM and RLM. The amounts of **2** found after 3 h incubation time ranked unanimously as follows: RLM > HLM > BLM > ARLM.

These findings shows that microsomes from human, rat and bovine liver are able to generate a broad spectrum of phase I metabolites when incubated with **1**, a common ingredient of cosmetics and constituent of flavoring preparations in foods and beverages. It needs to be investigated, however, if the differences found in liver microsomes from three different species are indicative of species-selective patterns of hepatic metabolism of **1** in intact cells or *in vivo*.

3'-Hydroxylation forming **2** was the major pathway of **1**. Alcohol **2** was shown to be the precursor of the α,β-unsaturated aldehyde **6**. This metabolite and the 1',2'-epoxide **12**, which

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could not be detected but can be assumed as precursor of the 1',2'-diol **7**, have the reactive capacity to bind to proteins and other hepatic macromolecules. The second major reaction of **1** in bovine and Aroclor-induced rat liver microsomes was found to be ring hydroxylation at position 6. This reaction was not or hardly detectable in microsomes from humans and non-induced rats. Surprisingly, formation of **8**, the allylic isomer of the 6-hydroxylated metabolite **5** was observed, bringing about the capability of formation of putative genotoxic secondary metabolites hydroxylated at the 1'-position. Future studies will be aimed at investigating the actual toxic potency of these reactive metabolites and their relevance of formation.

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Authorship Contributions

Participated in research design: Cartus and Schrenk

Conducted experiments: Cartus

Contributed new reagents or analytic tools: Cartus and Merz

Performed data analysis: Cartus

Wrote or contributed to the writing of the manuscript: Cartus, Merz and Schrenk

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Legends for Figures

Figure 1. Formation of metabolites and decrease of **1** during incubation of Aroclor1254-induced rat liver microsomes (ARLM) with **1** (500 μM; 0, 10, 20, 30, 60, 120, 180 min; 21 and 24 h) at a detection wavelength of λ = 280 nm with a reference wavelength of 340 nm. Time 0 means 5 min pre-incubation without NADPH-generating system. The chromatograms are representative for a series of n = 3 experiments.

Figure 2. Quantification of metabolite formation during incubation of liver microsomes with **1**. A: ARLM (rat, Aroclor-induced), B: RLM (rat non-induced), C: BLM (bovine), D: HLM (human). Initial concentration of **1** was 500 μM; concentration of **10** (internal standard) 500 μM. Values are means ± S.D. from three independent incubations. Symbols represent: - x - sum of metabolites; - □ - methylisoeugenol (**1**); - Δ - 3'-hydroxymethylisoeugenol (**2**); - ⬡ - 6-hydroxymethylisoeugenol (**5**); - ⬢ - 6-hydroxymethyleugenol (**8**);- ◁ - isoeugenol (**3**) + Isochavibetol (**4**) + 3-oxomethylisoeugenol (**6**) + 1',2'-dihydroxymethylisoeugenol (**7**).

Figure 3. HPLC separation of ARLM incubation with **2** (3 h, 500 μM). Detection of absorbance of eluting compounds at λ = 261 nm (A), λ = 280 nm (B). The chromatogram is representative for a series of n = 3 experiments.

Figure 4. Formation of metabolites and decrease of **5** during incubation of human liver microsomes (HLM) with **5** (100 μM; 10, 30, 60, 120 min) together with a NADPH-generating system, at a detection wavelength of λ = 280 nm with a reference wavelength of 340 nm.. The chromatograms are representative for a series of n = 3 experiments.

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Figure 5. Phase I metabolic pathways of methylisoeugenol (**1**) in liver microsomes.

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Tables

Table 1. Amounts of substrate, internal standard, and metabolites detected in microsomal (Aroclor-pretreated rat: ARLM; untreated rat: RLM; bovine: BLM; human: HLM) supernatants 2 h (or 10 min if indicated) after start of incubation. The amounts are expressed in % of the initial concentration of methylisoeugenol (**1**), which was 100 or 500 µM as indicated. Data represent means ± S.D. from n=3 incubations.

	ARLM		**RLM**		**BLM**		**HLM**	
compound	100 µM (10 min)	500 µM	100 µM	500 µM	100 µM	500 µM	100 µM	500 µM
internal standard (**10**)	97.8 ± 1.7	101.8 ± 5.9	99.6 ± 2.0	101.5 ± 5.4	99.3 ± 3.4	98.5 ± 4.1	100.8 ± 3.0	100.6 ± 2.0
methylisoeugenol (**1**)	11.4 ± 1.5	24.7 ± 5.0	36.5 ± 5.3	50.3 ± 15.4	8.3 ± 0.4	57.4 ± 6.1	n.d.	52.7 ± 5.7
3'-hydroxymethyleugenol (**2**)	14.2 ± 0.4	12.6 ± 1.2	26.2 ± 7.4	39.7 ± 7.7	24.2 ± 1.5	19.8 ± 0.8	33.9 ± 3.2	30.1 ± 2.5
isoeugenol + isochavibetol (**3+4**)	5.8 ± 0.0	6.9 ± 3.5	4.5 ± 0.2	2.7 ± 0.6	1.8 ± 0.2	1.2 ± 0.0	5.0 ± 0.4	3.6 ± 0.3
6-hydroxymethylisoeugenol (**5**)	34.5 ± 0.9	15.6 ± 0.9	n.d.	n.d.	3.3 ± 1.1	7.5 ± 0.2	n.d.	n.d.
3'-Oxomethylisoeugenol (**6**)	0.3 ± 0.0	0.5 ± 0.2	9.0 ± 1.1	2.1 ± 0.0	2.7 ± 0.7	0.7 0.2	7.0 ± 3.1	3.0 ± 1.1
1',2'-dihydroxymethylisoeugenol (**7**)	2.8 ± 0.6	3.2 ± 1.1	n.d.	n.d.	2.2 ± 0.1	2.3 ± 0.1	7.0 ± 1.6	1.0 ± 0.1
6-hydroxymethyleugenol (**8**)	n.d.	12.1 5.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
sum of metabolites	57.6 ± 2.7	50.9 ± 3.2	39.7 ± 2.8	44.6 ± 18.9	34.2 ± 2.8	31.5 ± 17.1	52.9 ± 4.2	37.6 ± 17.4

n.d. = not detectable

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Figure 1

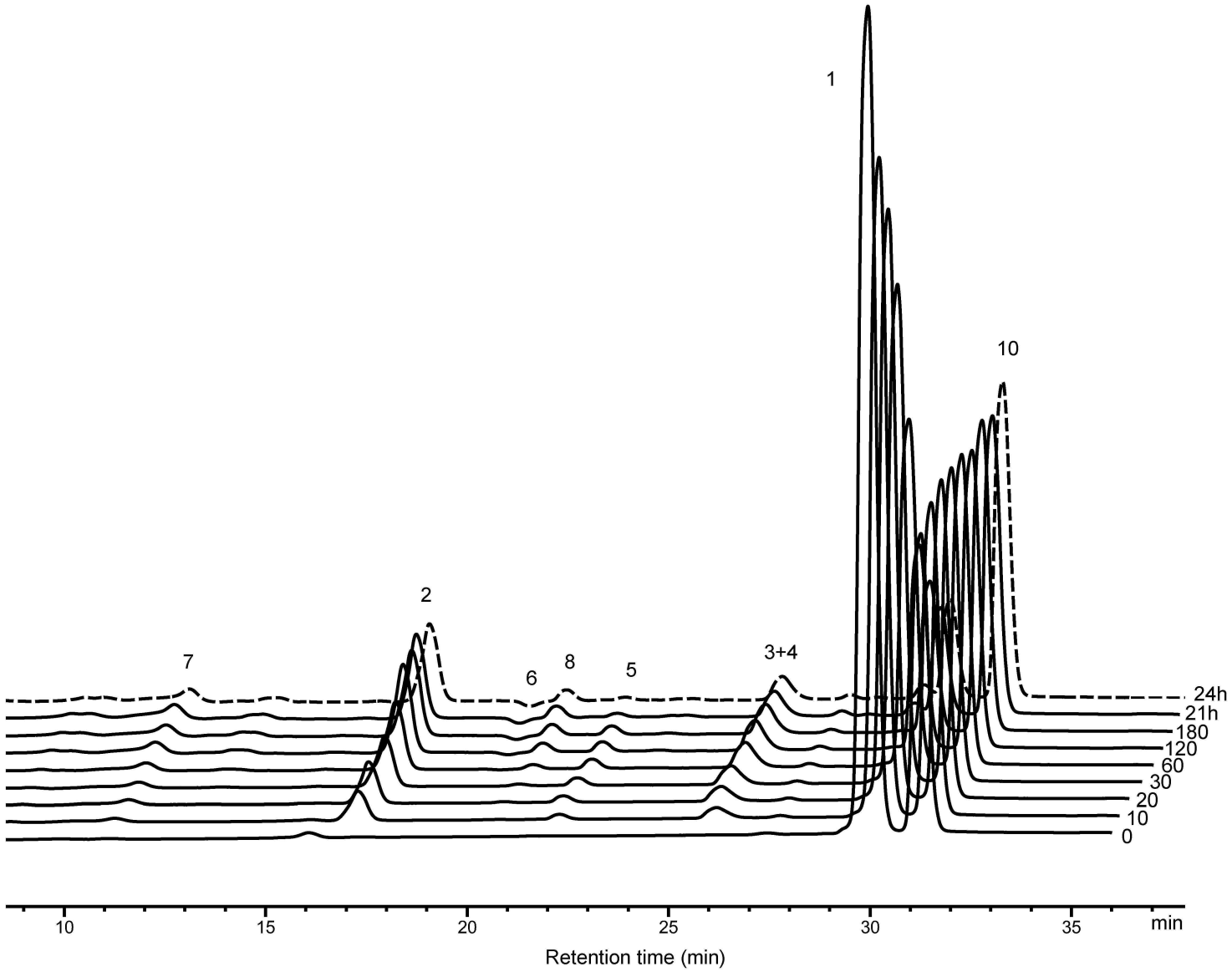

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Figure 2

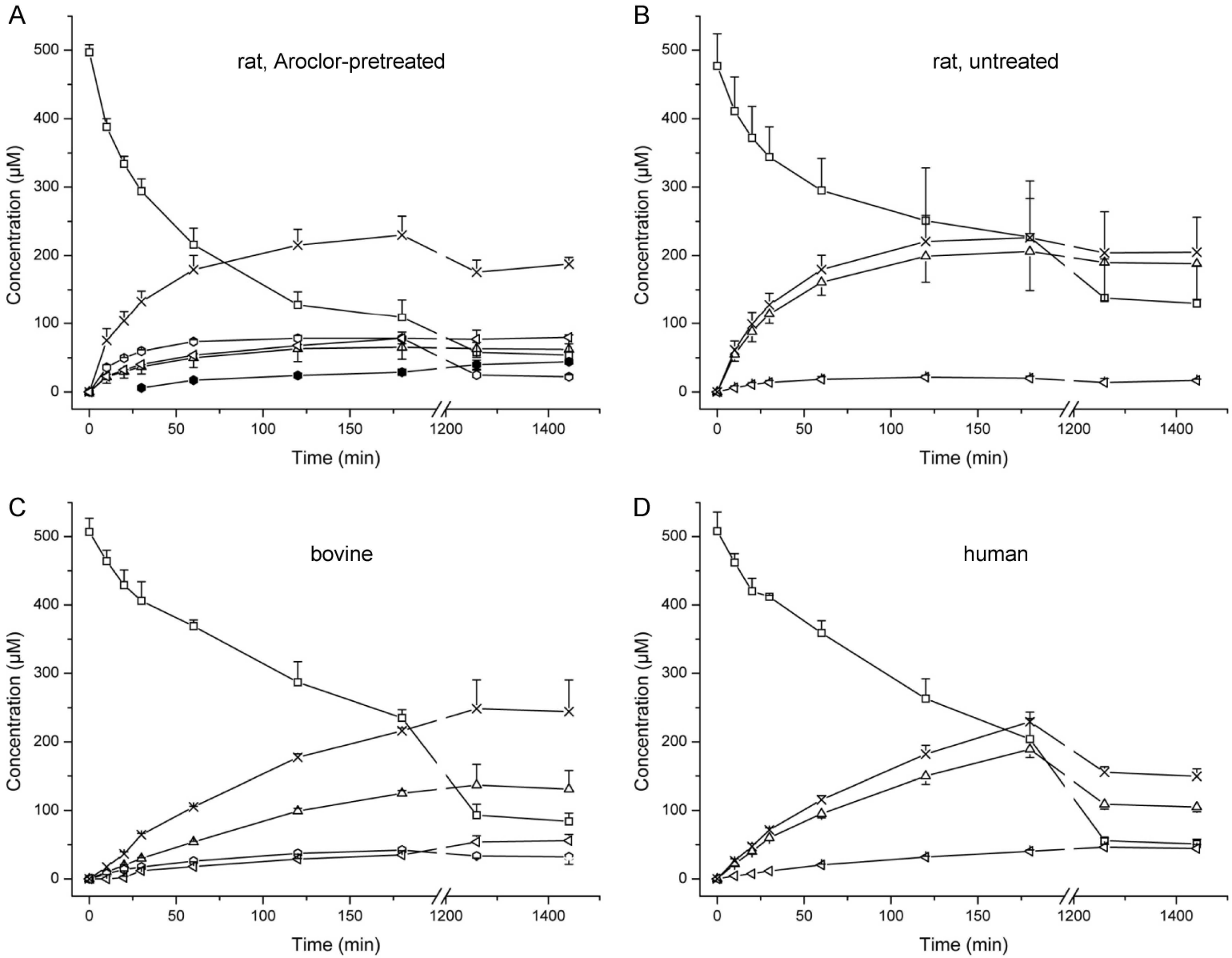

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Figure 3

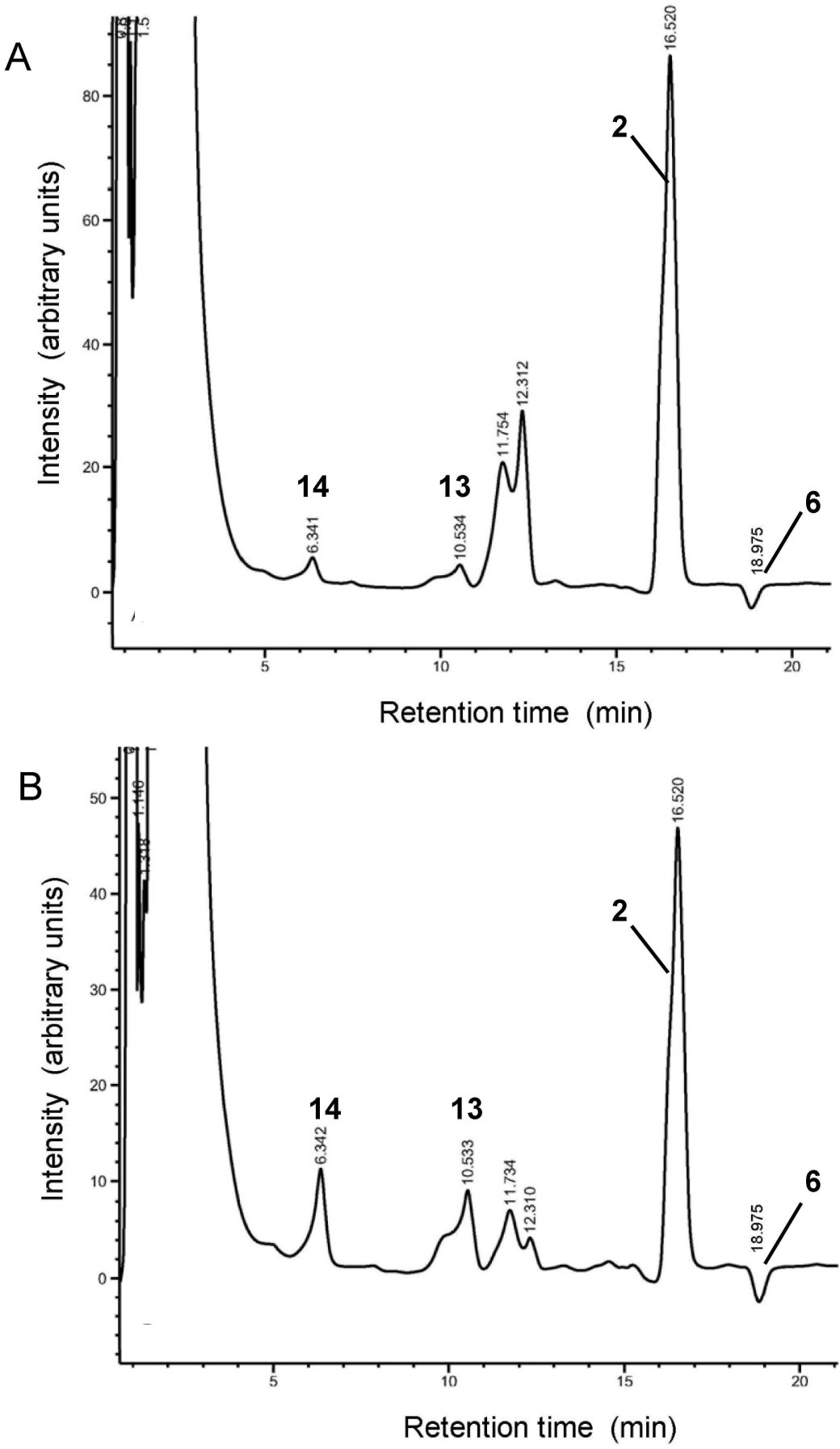
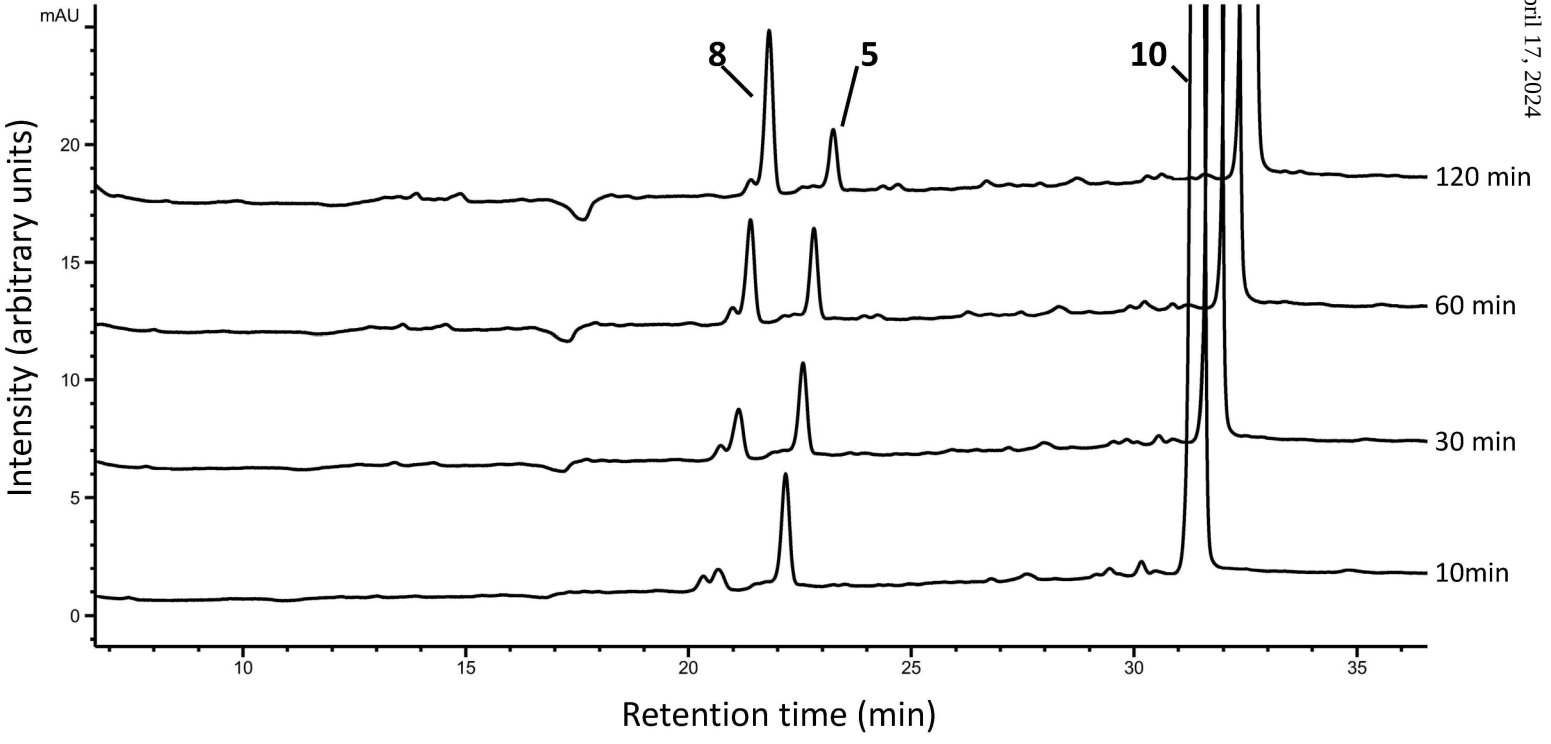
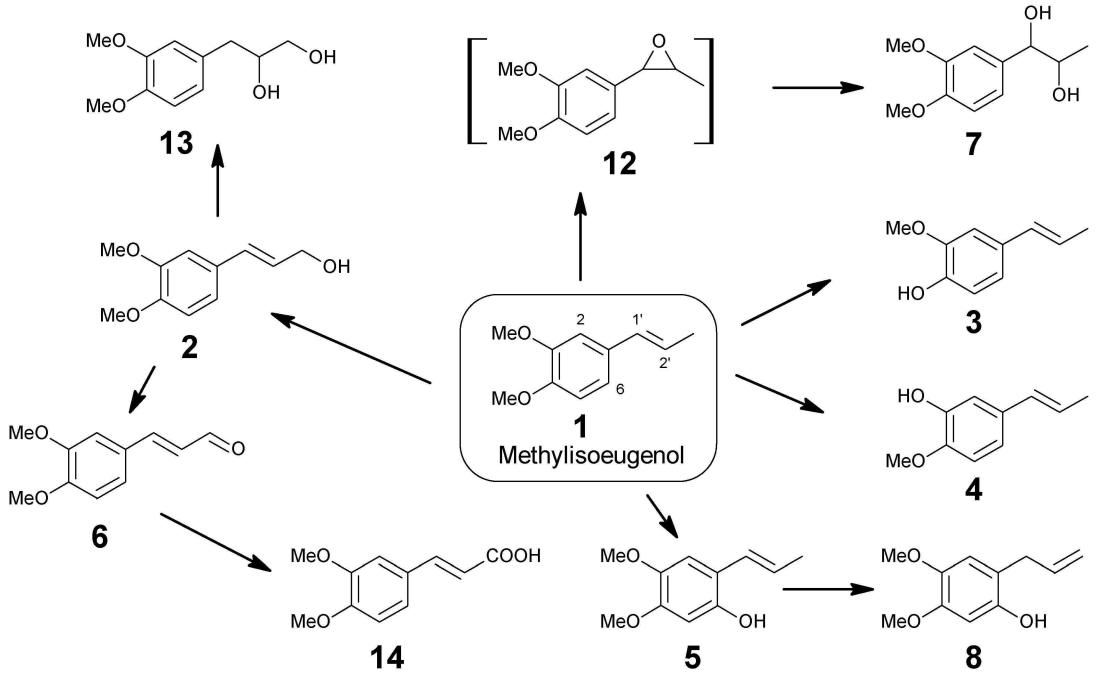

A
Intensity (arbitrary units)
Retention time (min)
16.520
2
14
13
6
6.341
10.534
11.754
12.312
18.975
B
Intensity (arbitrary units)
Retention time (min)
16.520
2
14
13
6
6.342
10.533
11.734
12.310
18.975

Figure 4



urnals on April 17, 2024

Fig. 5


MeO
MeO
OH
OH
13
MeO
MeO
O
12
MeO
MeO
OH
OH
7
MeO
MeO
OH
2
MeO
HO
3
2
1'
2'
6
MeO
MeO
1
Methylisoeugenol
HO
MeO
4
MeO
MeO
O
6
MeO
MeO
COOH
14
MeO
MeO
OH
5
MeO
MeO
OH
8