DOI 10.1002/mnfr.201000394 *Mol. Nutr. Food Res.* 2011, *55*, 644–653

RESEARCH ARTICLE

Relative bioavailability of coumarin from cinnamon and cinnamon-containing foods compared to isolated coumarin: A four-way crossover study in human volunteers

Klaus Abraham, Michael Pfister, Friederike Wöhrlin and Alfonso Lampen

Federal Institute for Risk Assessment, Berlin, Germany

Received: August 20, 2010
Revised: October 15, 2010
Accepted: October 29, 2010

Scope: Cassia cinnamon contains high levels (up to 1 %) of coumarin. Heavy consumption of this spice may result in a dose exceeding the tolerable daily intake (TDI). In this context, the question was raised whether coumarin in the plant matrix of cinnamon has the same bioavailability as isolated coumarin.

Methods and results: A four-way crossover study was performed, in which the same dose of 12 mg coumarin was administered in different formulations to 24 healthy volunteers. The relative extent of absorption measured as urinary excretion of the main metabolite 7-hydroxycoumarin (7OHC) was found to be 62.8% for isolated coumarin in a capsule (reference), 56.0% for cinnamon in capsules, 66.1% for cinnamon tea, and 54.7% for cinnamon in rice pudding (means, n = 23, observation period 8 hours). Additionally, 7OHC plasma levels were measured for 105 minutes after administration and revealed a fast absorption of coumarin from cinnamon tea leading to the highest peak concentrations.

Conclusion: The relative extent of absorption of coumarin from powder of cassia cinnamon is only slightly lower than that of isolated coumarin. Therefore, the TDI of coumarin can be used for risk assessment of coumarin exposure from cinnamon-containing meals.

Keywords:
Bioavailability / Cinnamon / Coumarin / CYP2A6 / Matrix effects

1 Introduction

Coumarin (1,2-benzopyrone, CAS No. 91-64-5, molar mass 146.14 g/mol) is a naturally occurring constituent of many plants with a pleasant spicy odour of fresh hay, woodruff, or vanilla. Its use as a flavouring has an up-and-down history. Coumarin was isolated from tonka beans in 1822 and, following its chemical synthesis in 1868, it was used as a flavouring substance until the middle of the last century [1]. Following the discovery of its hepatotoxic properties in laboratory animals, the use of isolated coumarin as a food flavouring substance was prohibited for the first time in the USA in 1954. Subsequently, coumarin was also found to be carcinogenic [2], and until the 1990s, it was not possible to rule out a genotoxic mechanism of action. After reviewing new experimental data in 2004, the European Food Safety Authority concluded that coumarin does not bind covalently to DNA, supporting a nongenotoxic mode of action. This made it permissible to derive a tolerable daily intake (TDI), which was done for the first time using animal data on hepatotoxicity [3]. The TDI of 0.1 mg/kg body weight daily was confirmed by the Federal Institute for Risk Assessment (BfR) 2 years later, using human data from coumarin

Correspondence: Dr. Klaus Abraham, Federal Institute for Risk Assessment (BfR), Department of Food Safety, Thielallee 88-92, 14195 Berlin, Germany
E-mail: klaus.abraham@bfr.bund.de
Fax: +49-30-8412-3763

Abbreviations: **AUC,** area under the plasma concentration time curve; **BfR,** Bundesinstitut für Risikobewertung; c_{max}, maximum concentration; **CYP,** cytochrome P450; **7OHC,** 7-hydroxycoumarin; **TDI,** tolerable daily intake; t_{max}, time of occurrence of maximum concentration; **VC,** variation coefficient

administration as a medicinal drug [4, 5] (www.bfr.bund.de/cm/245/consumers_who_eat_a_lot_of_cinnamon_currently_have_an_overly_high_exposure_to_coumarin.pdf).

A few years ago, high coumarin levels of up to 100 mg/kg were discovered in typical German Christmas cookies with high cinnamon content, indicating that coumarin exposure of heavy consumers of food spiced with cassia cinnamon may exceed the TDI [5]. Other edible plants and fruits may also contain coumarin; however, the concentrations are much lower [6] than those in cassia cinnamon: median levels are about 3000 mg/kg, though maximum levels were found to be nearly 9000 mg/kg in Germany [5, 7, 8] and even higher in other countries [9].

During the public discussion on coumarin and cinnamon, the question was raised whether coumarin in the plant matrix of cinnamon has the same toxicity as the isolated compound used in animal experiments and human medicine [10]. This is a general question, as foods usually have a very complex composition, and any particular substance is subject to potential interaction with a large number of other substances. These interactions may include effects on the liberation and absorption characteristics. In particular, this may significantly alter bioavailability of the substance. Furthermore, metabolism and excretion of a particular compound may be affected by other food constituents as well, thus also influencing its efficacy, for instance by activating or deactivating enzymes of the organism or the intestinal flora [11] (www.dfg.de/download/pdf/dfg_im_profil/reden_stellungnahmen/2006/sklm_natinh_en_05092006.pdf). Depending on the mechanism of action, this could result in the toxicity being unchanged, reduced, or even increased [12]. Such interactions can rarely be predicted. Therefore, the effect of the respective food matrix needs to be investigated experimentally on a case-by-case basis.

Apart from possible matrix effects, toxicity may also be influenced by different kinetics, following bolus or dietary application, resulting in higher peak levels in the former and in gradual uptake with lower peak levels in the latter. Though this often makes a big difference in animal experiments (application by gavage once a day *versus* addition to diet), it is generally less relevant in humans (*e.g.* oral medication *versus* intake with a meal). However, during the discussions in 2006 in the European Union, it was argued that the use of human data on hepatotoxicity following the medicinal administration of coumarin [13, 14] would overestimate the risk, as this is a bolus administration with kinetics not comparable to those of dietary consumption.

The aim of the present study was to answer the questions on kinetics addressed above (plant matrix, bolus *versus* dietary exposure) and hence to improve risk assessment of coumarin in cinnamon-containing foods. The basic kinetic parameters that describe bioavailability and bioequivalence of different formulations of a compound are the extent and velocity of absorption [15] (http://www.ema.europa.eu/pdfs/human/qwp/140198enrev1fin.pdf). To assess these parameters for coumarin in cinnamon, we performed a study in human volunteers to compare the extent of absorption and plasma peak levels of the isolated substance to that of three different formulations of cinnamon containing the same dose of coumarin. Isolated coumarin was directly compared with powdered cassia cinnamon using the same application method, capsules taken in a fasting state. As a representative of cinnamon-containing food, cinnamon was added to rice pudding, and for comparison with a cinnamon-containing drink (fluid matrix, *e.g.* teas such as Yogi Tea®), an aqueous cinnamon extract was prepared.

2 Materials and methods

2.1 Study design

In order to compare the bioavailability of isolated coumarin to that of coumarin from cinnamon-containing foods, a four-way crossover design involving 24 volunteers was chosen, following the "guideline on the investigation of bioequivalence" in case of medicinal products [15]. We compared the following four administrations, all given orally: isolated coumarin in capsule (way A, reference), powdered cinnamon in capsules (way B), an aqueous cinnamon preparation (way C), and cinnamon powder in rice pudding (way D). As coumarin has a very strong first-pass effect in the liver with only a low percentage reaching the systemic circulation [16, 17], we chose its main metabolite in humans, 7-hydroxycoumarin (7OHC, umbelliferone, CAS No. 93-35-6, molar mass 162.14 g/mol), as a measure of relative bioavailability. The conversion of coumarin to 7OHC is catalyzed by cytochrome P450 2A6 (CYP2A6) [18]. The compound and its phase II metabolite 7OHC glucuronide are rapidly excreted *via* the kidneys [19]; therefore, the total amount of 7OHC (free and bound as glucuronide) in urine quantitatively collected following the intake of coumarin is an indirect measure of the relative extent of absorption if compared for the different study applications on an individual basis.

In addition, the level of 7OHC (free and bound as glucuronide) was measured in blood plasma during the first 105 min after ingestion in order to monitor peak concentrations (c_{max}, maximum concentration) and time of their occurrence (t_{max}) as surrogate markers of these parameters for coumarin. At the dose of 12 mg used in the study, coumarin itself was measured at levels up to 16 ng/mL in pooled plasma samples using gas chromatography MS (unpublished data). However, at these levels the method was not found to be reliable enough for quantification of coumarin in blood plasma.

2.2 Volunteers

In order to assure high compliance, volunteers were recruited from the scientific staff of the BfR. They had to be healthy non-smokers of European origin who were not

taking medicinal drugs possibly influencing the kinetics of coumarin and its metabolites. Along with the experimental blood samples taken on the first day, extra samples were collected for measurement of liver enzymes, creatinine, and cellular blood parameters (blood count); all results obtained were within the normal range. The study group consisted of 12 nonpregnant female and 12 male participants with a mean age of 39.2 years (median 34.5, range 26–60), mean height of 1.75 m (median 1.73, range 1.56–1.96), mean body mass of 71.6 kg (median 70.0, range 49–102), and mean body mass index of 23.2 kg/m^2 (median 22.7, range 19.2–29.4). Four of the 12 females (33%) used oral contraceptives. Participants were instructed not to eat any cinnamon-containing foods or to use any coumarin-containing cosmetics (*e.g.* body lotion or aftershave) at least 3 days before investigation. In addition, no grapefruit was allowed during this period to avoid inhibition of CYP2A6 [20]. All participants gave informed consent in writing; the study protocol was approved by the ethics committee of the Charité – Universitätsmedizin Berlin (EA4/078/08).

2.3 Coumarin and cinnamon formulations

A coumarin dose of 12 mg was chosen as a compromise between a realistic (not too high) amount of cinnamon intake (*i.e.* 2 g, see below) and analytical detection limits. This dose is twice the TDI of 6 mg in a person with a body mass of 60 kg, the maximum that can be eaten daily with food for a lifetime. The European Food Safety Authority concluded in 2008 that exposure to coumarin resulting in an intake three times higher than the TDI for 1–2 wk is not of safety concern [21]. Four different coumarin/cinnamon formulations were investigated (ways A–D, for overview see Table 1).

Coumarin in drug quality (DAB) was from Fagron (Barsbüttel, Germany). For way A, an amount of 12.0 mg of the compound was measured into telescoping two-part gelatin capsules (not resistant to gastric acid; size 2, volume 0.37 mL) directly on an analytical balance.

In order to minimize the amount of cinnamon needed to reach a coumarin dose of 12 mg, cinnamon powder with a relatively high coumarin level was used. For this purpose, the three samples with the highest levels were identified in a set of commercially available packages of powdered cassia cinnamon that had been analyzed earlier [8]. These three samples, from different suppliers, were pooled (total amount 320 g) and mixed using an overhead shaker for 5 h. HPLC analysis (see below) revealed a coumarin level of 6240 mg/kg (eight samples with duplicate preparation, variation coefficient (VC) 1.7%) and good homogeneity. This meant that the dose of 12 mg coumarin was contained in 1.92 g of this cinnamon. For way B, this amount was weighed into seven telescoping two-part gelatin capsules (size 1, volume 0.50 mL) consisting of the same material as that of the coumarin capsules. For way D, the same amount was added to a 200 g package of rice pudding (''Milchreis'', Müller, Fischach-Aretsried, Germany) and mixed. For way C, a cinnamon tea was prepared by boiling 160 g of the same cinnamon in 4.5 L distilled water for 30 min in a pot, stirring from time to time. The cinnamon/water mixture was then centrifuged in 50-mL tubes ($2000 \times g$, 15 min), and the supernatant was filtered three-fold using a Büchner funnel and a Weißband filter (type 589/2, pore size 4–12 μm). The total resulting volume was divided into volumes of 125 mL and filled into freezer bags. HPLC analysis (see below) carried out on every fifth sample revealed a uniform coumarin level of 96.0 mg/L (seven samples, VC 1.3%). The tea was stored at −80°C until the day before administration, then at 4°C overnight to thaw. The extraction rate of coumarin (cinnamon powder to tea) was 38.5%.

HPLC analysis of cinnamon powder and cinnamon tea was carried out according to the methods described in detail elsewhere [8]. Briefly, all the chemical products and solvents were of the highest grade available and acquired from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Cinnamon powder (100 mg) was extracted twice using methanol and analyzed in triplicate. Methanol extracts and aqueous tea preparations were filtered through centrifugal filters before injection into HPLC. The HPLC system used was an Agilent (Waldbronn, Germany) 1100 HPLC system (quaternary pump, degasser, and autosampler) with UV detector. Analysis was performed on a reversed-phase column (Phenomenex Aqua 3 μ C18 125A, 150 × 2 mm; Phenomenex, Aschaffenburg, Germany) at 35°C using mobile-phase 5 mmol ammonium acetate buffer (pH 5.0)/ACN in a gradient program (flow rate: 350 mL/min, detection at a wavelength of 274 nm). The injection volume was 10 μL. The analytical method was validated in-house assessing repeatability, linearity, limit of detection, and limit of quantification.

Table 1. Overview on the four study ways of administration, all containing 12 mg coumarin

Way	A Coumarin in capsule	B Cinnamon in capsules	C Cinnamon tea	D Cinnamon with rice pudding
Amount	12 mg coumarin in one capsule	1.92 g cinnamon (coumarin content 6240 mg/kg) in seven capsules	125 mL cinnamon tea (coumarin content 96.0 mg/L)	1.92 g cinnamon (coumarin content 6240 mg/kg) in 200 g rice pudding
Rationale	Reference	Direct comparison with A	Fluid matrix	Typical meal

2.4 Investigation and sample collection

Volunteers fasted overnight and came to the test location at about 8:00 a.m. without breakfast. For repeated blood sampling, an intravenous catheter (Introcan Safety, B. Braun Melsungen AG, Germany) closable by a mandrin was placed in a vein of the antecubital fossa. For plasma collection, a whole-blood sample was drawn (9 mL vial, 16 IU Li-Heparin/mL blood, Sarstedt S-Monovette 02.1065, Nümbrecht, Germany); the glucose level was checked using a glucose meter for diabetics. Participants also provided a urine sample, before one of the four study administrations was given: the capsule(s) (ways A and B) were swallowed with as little tap water as possible (in order to be quickly broken up by gastric acid), followed by the remaining amount of 200 mL 10 min later. The tea (way C, 125 mL portion) was warmed to room temperature and drunk within about 1 min. The rice pudding with cinnamon (way D) was eaten within about 3 min. Both tea and rice pudding were followed by drinking of 100 mL tap water (total amounts of fluid intake were planned to be comparable on the 4 days of investigation). A further 200 mL of tap water was drunk after 90 min; apart from this, no other foods or beverages were consumed within the first 3 h. After this time, the participants were allowed to eat and drink *ad libitum*. During the test day, participants were mainly remained in a sitting position working at a computer (low physical activity level). Whole blood (9 mL) was collected again at 15, 30, 45, 60, 75, 90, and 105 min after application; vials were centrifuged at $2000 \times g$ for 10 min, and the supernatant plasma was divided into three samples of at least 1.2 mL. Urine was collected quantitatively in 2-h fractions until 8 h after application. Each fraction was weighed and three 3-mL aliquots were taken. All samples were stored in cryo vials at −80°C until analysis.

The study was performed between April and August 2009. The intervals between tests on each volunteer were between 1 and 2 wk. Each participant received the administrations in a different sequence (*e.g.* ways C, B, A, and D; *i.e.* one of the 24 sequences possible).

2.5 Analysis of 7OHC in human samples

Unless stated otherwise, all chemical products and solvents were of the highest grade available. 7OHC was purchased from Sigma-Aldrich Chemie GmbH; a stock solution (1 mg/mL) was prepared in methanol (HPLC grade), Promochem, Wesel, Germany) and diluted daily with ultrapure water to a concentration of 10 and 1 µg/mL, respectively. Deuterated 7OHC (7-hydroxycoumarin-2,3,5,6,8-d_5) was purchased from TLC PharmaChem (Mississauga, Ontario, Canada) and used as an internal standard; a stock solution (1 mg/mL) was prepared in ACN (Gradient grade, VWR International, Dresden, Germany) and diluted daily to a concentration of 10 µg/mL with ultrapure water as a working solution. Ammonium acetate and glacial acetic acid were purchased from Merck (Darmstadt, Germany). β-Glucuronidase from *Escherichia coli* K12 (Roche, Mannheim, Germany) was diluted 1:1 with ultrapure water.

After thawing at room temperature, the samples of blood plasma were centrifuged at $3200 \times g$. A volume of 250 µL was transferred to glass sample tubes, and 7OHC-d_5 was added as an internal standard to a final concentration of 1000 ng/mL. To adjust a suitable pH-value, 1000 µL ammonium acetate buffer ($c = 1$ mol/L, adjusted with glacial acetic acid to pH 6.5) was added. After adding 10 µL glucuronidase solution, the samples were incubated for 2 h at 37°C in a water bath. The samples were then diluted with 1 mL ultrapure water, mixed up by vortexing, and cleaned up by SPE on Phenomenex Strata-X tubes (1 mL, 30 mg). The tubes were conditioned with 1 mL methanol, and then equilibrated with 1 mL buffer solution (250 µL ultrapure water+750 µL ammonium acetate buffer ($c = 1$ mol/L)). Samples were loaded and the tubes were washed two times with 1 mL ultrapure water. The tubes were then dried with nitrogen. Finally, the samples were eluted with 2×0.5 mL methanol/water/formic acid (80/20/0.2). The collected extracts were combined and subsequently evaporated with nitrogen to a volume of about 250 µL. The concentrated extracts were transferred into glass vials and injected for HPLC-MS/MS analysis. All samples were prepared and analyzed in duplicate.

After thawing at room temperature, urine samples were centrifuged at $3200 \times g$. Aliquots of 1000 µL were used for sample preparation. 7OHC-d_5 was added as an internal standard to a final concentration of 1000 ng/mL. The urine samples were treated with glucuronidase as published [22] with slight modifications and then extracted twice each with 2 mL diethyl ether (SeccoSolv, Merck). The collected extracts were combined and evaporated with nitrogen until dryness. The residue was reconstituted with 1000 µL mobile phase and transferred to glass vials for HPLC-MS/MS analysis. All samples were prepared and analyzed in duplicate.

Separation and quantification were carried out by LC-MS/MS an Agilent 1100 HPLC (binary gradient pump system and an automatic injection system) and an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). The separation was carried out under isocratic conditions (flow rate: 0.2 mL/min; eluent A (45%): ammonium acetate 10 mmol/L; eluent B (55%): 95% methanol/5% ACN). The column used for the separation was a Phenomenex Luna PFP(2) (2×100 mm, 3 µm particle diameter). The injection volume was 5 µL. The HPLC system was connected to the mass spectrometer through an ESI interface. The interface was heated to 275°C. Collision gas pressure was set to 12.0 psi, and the ionization voltage was −3500 V. The measurement was carried out with multiple reaction monitoring. 7OHC was measured at the masses m/z 161.20 (quadrupole 1) and m/z 133.00 (quadrupole 3). 7OHC-d_5 was measured at the masses m/z 166.20 (quadrupole 1) and m/z 138.00 (quadrupole 3). The collision energy for both paired masses was set to −28.0 V.

Matrix calibrations of analyte-free blood plasma and urine, spiked with the internal standard to a final concentration of 1000 ng/mL and 7OHC to give final concentrations from 0 to 3000 ng/mL, were carried out daily before the measurement of experimental samples. The 7OHC content was calculated on the basis of the 7OHC-d_5 internal standard. Results of single samples are averages of duplicate analyses. The analytical method was validated in-house assessing repeatability (VC = 3.6%), linearity, limit of detection (about 3.4 ng/mL), and limit of quantification (about 11 ng/mL). Within each analysis series, a quality assurance sample (pooled urine and pooled plasma, respectively) was analyzed in duplicate; the values of these measurements were monitored with a Shewhart control chart.

2.6 Evaluation and statistics

Urinary excretion of 7OHC for the four 2-h fractions was calculated from the data of concentration and urine weight of the corresponding sample, assuming a specific weight of 1. Analytical results below the limit of detection were set to zero. The respective values of the different administrations were tested ($p < 0.05$) using a two-sided *t*-test for paired data. Calculations were performed using Microsoft® Office Excel 2003 and SPSS Version 12.0.

3 Results

The four investigations in the 24 volunteers were performed without complications or mistakes (*e.g.* incomplete collection of urine or missing blood samples due to occlusion of the intravenous catheter). For every administration, all volunteers started in the morning in a fasting state, confirmed by measuring blood glucose levels, which were all within the fasting normal range. No 7OHC was detectable in the 96 blood and 96 urine samples taken before administration. Therefore, all participants were included in the first evaluation and showed fast urinary 7OHC excretion as expected, but in one male participant (no. 20) a delayed excretion was observed for all four administrations. However, his corresponding 7OHC blood levels were within the range of the other participants. As a nearly complete urinary 7OHC excretion within the observation period of 8 h is a prerequisite for this study of bioavailability, this volunteer's data were excluded from the final evaluation, and the following results are from 23 test persons (12 female and 11 male).

3.1 Plasma levels of 7OHC

Plasma levels of 7OHC within the first 105 min after application are shown in Fig. 1. The fastest increase was observed for cinnamon tea (way C), showing mean levels (± SD) of 178 ± 62 and 340 ± 68 ng/mL after 15 and 30 min, respectively; after this time, mean plasma levels were already started to decrease. A slower increase was observed for cinnamon in rice pudding (way D), showing a mean level of 42 ± 21 ng/mL after 15 min and a mean maximum level of 188 ± 48 ng/mL after 45 min. For the two administrations in capsule(s), most samples were below the limit of detection at 15 min; mean maximum levels of 221 ± 72 ng/mL for cinnamon (way B), and 190 ± 46 ng/mL for coumarin (way A) were reached after 60 and 75 min, respectively. At the end of the observation period (105 min), mean 7OHC levels were highest for the capsule administrations (151 ± 41 ng/mL for way A and 137 ± 45 ng/mL for way B), whereas mean levels for cinnamon in rice pudding (way D, 107 ± 32 ng/mL) and for cinnamon tea (way C, 97 ± 33 ng/mL) already were distinctly lower.

Variation of the individual levels was high during the first period of absorption, especially with administrations given in capsules: 30 min after application, the VCs were 80, 95, 20, and 29% for coumarin capsule, cinnamon

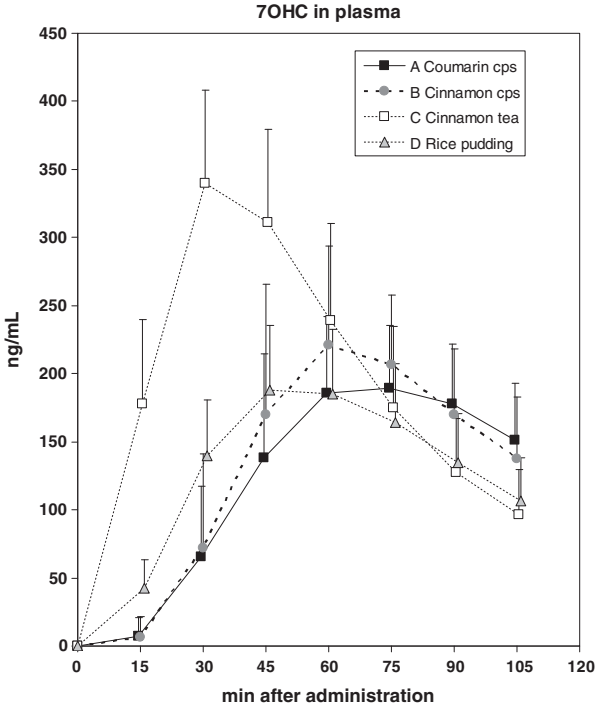

Figure 1. Time course of mean 7OHC levels in plasma during 105 min after administration of the four study administrations. Plasma samples were treated with β-glucuronidase before analysis. Symbols of the different applications are slightly shifted by 0.5 min to avoid overlapping of SDs displayed as whiskers ($n = 23$). Compared with the means of way A as reference, means of the other applications were found to differ significantly (two-sided *t*-test for paired data) in case of way B at 60 and 75 min only ($p < 0.01$), in case of way C at all time points ($p < 0.001$) apart from 75 min, and in case of way D at all time points ($p < 0.01$) apart from 60 min.

Table 2. Plasma levels of 7OHC: evaluation of maximum values (c_{max}) and the corresponding time of their occurrence (t_{max}) observed in individual subjects ($n = 23$; in contrast to data of this table, Fig. 1 shows mean ± SD values for the different times of blood sampling)

Way	Mean ± SD	Median	Minimum	Maximum
A (coumarin capsule)				
c_{max} (ng/mL)	203 ± 52	202	96	341
t_{max} (min)	70 ± 15	90	75	45
B (cinnamon capsules)				
c_{max} (ng/mL)	240 ± 65	240	133	358
t_{max} (min)	64 ± 12	75	60	60
C (cinnamon tea)				
c_{max} (ng/mL)	347 ± 68	343	205	479
t_{max} (min)	33 ± 6	45	30	30
D (cinnamon rice pudding)				
c_{max} (ng/mL)	197 ± 49	200	74	290
t_{max} (min)	50 ± 11	30	60	45

Plasma samples were treated with β-glucuronidase before analysis. The four mean values of c_{max} were significantly different from each other (two-sided *t*-test for paired data, $p < 0.001$), but not those of ways A and D.

capsules, cinnamon tea, and cinnamon in rice pudding, respectively. Maximum plasma levels (c_{max}) observed in individual volunteers (independently of time after administration) are summarized in Table 2. Individual ratios of c_{max} of the three cinnamon administrations (ways B–D) to c_{max} of the coumarin administration (way A as reference) had a mean ± SD (90% confidence interval) of 1.20 ± 0.25 (0.82–1.81), 1.80 ± 0.32 (1.30–2.31), and 0.99 ± 0.22 (0.54–1.45), for way B, way C, and way D, respectively.

3.2 Excretion of 7OHC in urine

The amount of urinary excretion of 7OHC was evaluated for the four 2-h periods (Table 3) and calculated as a percentage of coumarin dose administered. As shown in Fig. 2, a large fraction was already excreted within the first 2 h after administration. The mean 7OHC excretion (± SD) in this period as a percentage of the total 7OHC excretion within the observation period of 8 h was found to be 58.8 ± 9.0% (coumarin capsule), 70.5 ± 8.1% (cinnamon capsules), 73.4 ± 5.9% (cinnamon in rice pudding), and 80.3 ± 5.4% (cinnamon tea). These differences reflect the different velocities of coumarin absorption (Fig. 1). The somewhat delayed absorption of coumarin from capsule (way A) resulted in the lowest mean 7OHC excretion in the first urine fraction, followed by the highest mean excretion in the subsequent fractions (Fig. 2). In the last fraction collected (6–8 h), a very low mean percentage of the dose applied (± SD) was observed for all administrations (1.0 ± 0.8, 0.8 ± 0.5, 0.8 ± 0.5, and 0.8 ± 0.4% for ways A–D, respectively), justifying the neglect of 7OHC remaining in the organism after 8 h. Therefore, the following calculations are not biased by the speed of absorption.

Table 3. Urinary excretion of 7OHC (mean ± SD, median, minimum, and maximum) within the four 2-h periods as well as the total period (0–8 h), given for each of the four study administrations ($n = 23$)

Way	Hours	Mean ± SD (mg)	Median (mg)	Range (mg)
A	0–2	4.93 ± 0.99	4.88	3.26–6.79
	2–4	2.68 ± 0.54	2.77	1.58–3.59
	4–6	0.61 ± 0.28	0.55	0.22–1.34
	6–8	0.14 ± 0.11	0.13	0.00–0.38
	0–8	8.36 ± 0.85	8.44	6.02–9.44
B	0–2	5.25 ± 0.80	5.39	3.52–6.50
	2–4	1.67 ± 0.49	1.61	0.89–2.96
	4–6	0.43 ± 0.22	0.35	0.12–1.00
	6–8	0.10 ± 0.06	0.10	0.00–0.24
	0–8	7.45 ± 0.78	7.68	5.15–8.54
C	0–2	7.07 ± 0.79	6.90	5.40–8.56
	2–4	1.30 ± 0.39	1.21	0.84–2.25
	4–6	0.32 ± 0.11	0.32	0.16–0.57
	6–8	0.11 ± 0.07	0.09	0.01–0.28
	0–8	8.80 ± 0.74	8.99	6.51–9.71
D	0–2	5.34 ± 0.72	5.26	3.72–6.84
	2–4	1.48 ± 0.33	1.48	0.86–2.26
	4–6	0.35 ± 0.12	0.36	0.12–0.62
	6–8	0.10 ± 0.06	0.10	0.00–0.23
	0–8	7.28 ± 0.75	7.34	4.84–8.43

The dose of 12 mg coumarin corresponds to an amount of 13.31 mg 7OHC. Urine samples were treated with β-glucuronidase before analysis.

The relative extent of absorption was evaluated as total (cumulative) urinary 7OHC excretion within 8 h. Mean ± SD was 62.8 ± 6.3% for coumarin in capsule (way A),

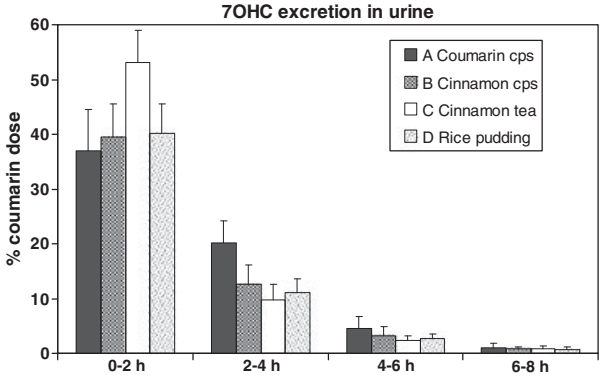

Figure 2. Mean and SD of urinary excretion of 7OHC, calculated as percentage of the coumarin dose applied, for the four study administrations during the first 8 h divided in four 2-h periods ($n = 23$). Urine samples were treated with β-glucuronidase before analysis. Compared with the means of way A as reference, means of the other applications were found to be significantly higher ($p < 0.05$, two-sided *t*-test for paired data) in case of ways C and D for the period 0–2 h, and significantly lower ($p < 0.01$) in case of ways B–D for the periods 2–4 and 4–6 h.

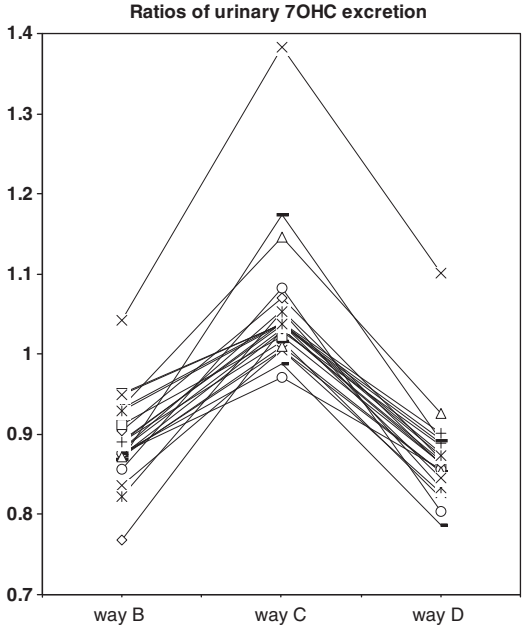

Figure 3. Individual ratios of urinary 7OHC excretion within 8 h for the three cinnamon administrations to that of the coumarin administration in capsule. For the 23 participants, different symbols were used and connected with a line. Urine samples were treated with β-glucuronidase before analysis.

56.0 ± 5.9% for cinnamon in capsules (way B), 66.1 ± 5.5% for cinnamon tea (way C), and 54.7 ± 5.6% for cinnamon in rice pudding (way D). All differences were found to be significant.

Individual results are shown in Fig. 3, calculated as the ratio of urinary 7OHC excretion of the different cinnamon administrations (ways B–D) to that of the reference coumarin administration (way A). All but one participant had ratios below 1.0 with cinnamon capsules (way B) and cinnamon in rice pudding (way D). The mean ratios ± SD (90% confidence intervals) were 0.89 ± 0.05 (0.78–1.02, way B) and 0.87 ± 0.06 (0.79–1.07, way D). On the contrary, only two participants had a ratio below 1.0 with cinnamon tea (way C); mean ratio ± SD was 1.06 ± 0.08 (90% confidence interval 0.97–1.34). In all volunteers, urinary 7OHC excretion was higher following coumarin administration in cinnamon tea than with cinnamon capsules or cinnamon in rice pudding.

4 Discussion

4.1 Study design

In pharmacology, relative bioavailability describes the extent and rate (velocity) by which a compound in a specific formulation becomes systemically available relative to a reference formulation of the same compound [15]. In the case of coumarin, the liver is the main target of toxicity [3, 5]. Following oral administration, almost all (> 94% [16]) of the dose is already metabolized during first pass after absorption and does not reach the systemic circulation. Therefore, the classical approach to determine bioequivalence after a single dose, measurement of area under the plasma concentration time curve (AUC), and c_{max}, is not useful for coumarin. However, coumarin's main metabolite in humans, 7OHC, can be used as an indirect measure of relative bioavailability of different coumarin formulations. It is rapidly excreted *via* kidney (mainly in its glucuronidated form), and the total 7OHC amount in urine is a relative measure for the extent of absorption of coumarin if the different formulations are compared intra-individually.

This concept makes sense if most of the coumarin administered is metabolized (by CYP2A6) to 7OHC. Individual mean urinary 7OHC excretion within 8 h (all four administrations) was between 42.3 and 67.3% of the coumarin dose applied in 23 subjects, with only three values below 57%. This was considered to be high enough to use 7OHC excretion as a measure of relative extent of absorption of coumarin. On the contrary, volunteer no. 20 excluded from the final evaluation had a mean urinary 7OHC excretion within 8 h (all four administrations) of only 25.0% of the dose. He had a deviating time pattern of excretion with a mean urinary 7OHC excretion of 7.0, 8.7, 5.2, and 4.1% of the dose within the periods 0–2, 2–4, 4–6, and 6–8 h, respectively (see Fig. 2 for comparison with the other participants), indicating incomplete excretion within 8 h. As his corresponding 7OHC blood levels were found to be in the range of the other participants (within the first 105 min after administration), this indicates a problem of urinary excretion, not of intestinal absorption or hepatic metabolism. In general, excretion rates of 7OHC are much higher than expected from glomerular filtration only, indicating

active tubular secretion of the 7OHC glucuronide [16]. These mechanisms are probably different in participant no. 20; further investigations are being done to clarify this phenomenon.

Besides urinary 7OHC excretion as measure for the extent of absorption, plasma levels of 7OHC were measured during the first 105 min after administration, as the rate (velocity) of intestinal absorption and the resulting peak level of a compound (c_{max} at t_{max}) also is an important parameter of relative bioavailability. In addition, these measurements allowed a better understanding of results from urinary 7OHC excretion, as already demonstrated for participant no. 20. Measurements of 7OHC served as surrogate marker of coumarin itself, as the parent compound was not found to be reliably quantifiable using gas chromatography MS. Also, in view of coumarin's strong first pass effect and the fact that liver is the critical target organ of toxicity, measurements of coumarin levels in the portal vein would be necessary to obtain kinetic data most adequate in relation to possible hepatic toxicity. This, of course, is not possible in humans, but due to the strong first pass effect of coumarin, the time course of plasma 7OHC levels is expected to reflect time course of coumarin in the portal vein shortly after administration.

A crossover design was chosen as an established concept of pharmacological bioequivalence studies which makes use of the lower intra-individual variability compared with higher inter-individual variability in parallel designs [15]. In general, both extent and rate of absorption from the gastrointestinal tract are very complex and affected by many factors [23]. In order to keep the week-to-week variability in individuals, low, study conditions were chosen to control as many physiological factors of influence as possible (*e.g.* empty stomach, same start time in the morning, same amount of fluid intake, no other foods during the first 3 h, and uniform low level of physical activity). With these conditions and high compliance of the participants, intra-individual variability was found to be relatively low (Fig. 3).

4.2 Plasma levels of 7OHC

Plasma levels of 7OHC within 105 min after application of different formulations (Fig. 1) showed the typical time course expected after an oral intake of a compound. Absorption of coumarin from cinnamon tea (way A) was distinctly faster than that from the other three formulations, leading to a higher mean 7OHC plasma level already at the 30 min. This does not reflect a different extent of absorption, but different galenics: in the tea, coumarin is already dissolved in a relatively large volume (125 mL plus 100 mL tap water), and on an empty stomach it is expected to be quickly transported to small intestine. On the contrary, with capsule administrations (coumarin, way A, and cinnamon powder, way B) a time delay is caused by the process necessary to break down the capsule material, and to dissolve/suspend its contents; this delays the appearance of the maximum levels (75 and 60 min, respectively) and leads to relatively high variability 30 min after administration (VCs 80 and 95%, respectively, compared with 20% for cinnamon tea and 29% for cinnamon in rice pudding). With cinnamon in rice pudding (way D), absorption was faster than that of the capsule administrations, but without reaching a higher maximum mean value.

In animal experiments, a big difference in kinetics (especially in peak levels reached) can be expected between dietary application of a compound with feed and bolus application once daily [11]. In humans, however, such a difference may not be observed, if the compound is part of a specific meal eaten only once daily, and if the bolus application is a tablet or capsule with the same dose of the compound taken once daily. This is demonstrated for the time course of 7OHC plasma levels (Fig. 1), revealing no big difference between the typical cinnamon meal (way D) and the coumarin in a capsule (way A). If the peak level above a threshold is the relevant trigger for the effect and not the AUC [5], consumption of, *e.g.*, a cinnamon-containing meal with a certain coumarin dose eaten once daily could even be more critical than the intake of coumarin tablets two or three times daily containing the same total dose; with the same dose of coumarin consumed in the form of cinnamon tea (*e.g.* Yogi Tea®) drunk once daily, peak levels higher than those of a cinnamon meal are to be expected. The results of this crossover study invalidate the argument that human data on hepatotoxicity following the medicinal administration of coumarin [13, 14] would overestimate the risk because of bolus application.

The question of the dose metric (hepatic peak level or AUC) relevant for the hepatotoxic effect of coumarin also applies to a possible dermal coexposure from coumarin used as a fragrance in cosmetic products without regulatory limits (concentrations above 0.001% in ''leave-on'' and above 0.01% in ''rinse-off'' products have to be declared in the European Union according to directive 76/768/EWG). Dermal absorption of coumarin is high and may approach 100% depending on the formulation [24]. Due to slower absorption and the lack of a first pass effect, hepatic peak concentrations are expected to be much lower in case of dermal exposure compared with oral exposure to the same dose. In order to compare the kinetics of oral and dermal application, 12 of the volunteers participated in an additional investigation in which they were exposed to the same dose of coumarin applied dermally in an *eau de toilette*; the results will be published separately.

4.3 Relative extent of absorption

The four coumarin/cinnamon formulations were absorbed with different velocities, with slowest absorption for coumarin in capsule (Fig. 1), leading to a slightly delayed urinary 7OHC excretion within the first hours after application (Fig. 2 and Table 3). However, in the last urine

fraction (6–8 h), only a very small percentage of the dose (mean ≤ 1%) was found as 7OHC. Therefore, total urinary 7OHC excretion within 8 h is not expected to be significantly influenced by the velocity of absorption, and can be used as a measure of the extent of absorption of coumarin according to the considerations made above (Section 4.1).

The extent of absorption of coumarin was found to be highest for cinnamon tea, followed by coumarin in capsule and the two administrations with cinnamon. Mean values for the latter were not very different (56.0% of the dose excreted as 7OHC for cinnamon in capsules, 54.7% for cinnamon in rice pudding). Obviously, the extent of absorption of coumarin from cinnamon powder is not much influenced if mixed into rice pudding, but even this difference was statistically significant due to low intra-individual variation and the relatively high number of volunteers. Isolated coumarin given in a capsule had a higher mean extent of absorption (62.8% of the dose excreted as 7OHC). We can only speculate about the underlying mechanisms of lower bioavailability of coumarin from cinnamon powder; they may involve incomplete liberation from the matrix during intestinal passage or interference with other compounds during membrane passage. The mean extent of absorption of coumarin in cinnamon tea was surprisingly high (66.1% of the dose excreted as 7OHC), slightly higher even than that of coumarin in capsule. The result is not believed to be influenced by different galenics, leading to faster absorption of coumarin from cinnamon tea. Theoretically, it might be that components of the cinnamon matrix that interfere with absorption of coumarin are not transferred from cinnamon powder to the tea during preparation.

A general question in this context is the absolute oral bioavailability of coumarin. A study that compared oral (coumarin dissolved in propylene glycol) and intravenous administration concluded that it was completely absorbed [16, 17]. However, the oral and intravenous doses were different, and only four subjects were investigated, revealing individual ratios of the 7OHC AUC (oral to intravenous) of 0.99, 1.34, 1.68, and 0.89. Therefore, this result does not seem to be very reliable. Rautio *et al.* [19] investigated two subjects with the same dose of 5 mg coumarin and stated that ''roughly the same'' percentage of 7OHC was recovered both after oral and intravenous administration. If absorption of coumarin is nearly complete, the total amount of urinary 7OHC excretion (including its glucuronide) is roughly two-thirds of the coumarin dose (this study and epidemiological studies in Europe using coumarin to phenotype CYP2A6 [19, 25]). A further metabolite identified in humans is *o*-hydroxyphenylacetic acid (*via* 3,4-epoxidation), with a mean urinary excretion rate of about 4% reported for relatively high coumarin doses of 200 mg [26] and 1000 mg [27]. In our study, LC-MS/MS analysis of pooled plasma samples revealed maximum levels of *o*-hydroxyphenylacetic acid of about 28 ng/mL for cinnamon tea, *i.e.* about 8% of the levels of 7OHC; 3-hydroxycoumarin as possible further metabolite was not detectable (unpublished data). Other major metabolic pathways in humans are currently not identified, not even in Asian populations with high frequencies of people deficient in the CYP2A6 pathway to 7OHC [28]. The fraction of the dose of coumarin which remained unidentified may be excreted *via* bile or as phase-II conjugates of 7OHC other than the glucuronide. There are currently no hints that the metabolism of coumarin may be influenced by other ingredients of cinnamon.

5 Conclusions for risk assessment of coumarin in cassia cinnamon

Compared with coumarin in capsule as reference, the relative extent of absorption of coumarin was found to be lower with cinnamon powder administered in capsules or with rice pudding. Although the difference is statistically significant, it is not important in terms of risk assessment of coumarin in cinnamon-containing foods (relative extent of absorption of coumarin in cinnamon rice pudding is 87% of that of isolated coumarin in capsule). Using the classical 0.80–1.25 limits for the 90% confidence intervals in pharmacology [15], criteria of bioequivalence were nearly reached for urinary excretion of 7OHC as surrogate marker of the AUC in case of cinnamon powder and cinnamon in rice pudding. Furthermore, peak levels of 7OHC as a surrogate marker for coumarin itself were in the same range for coumarin in capsule and coumarin in a cinnamon-containing meal (rice pudding); in this case, the faster absorption of the latter compensates the slightly lower extent of absorption. Therefore, the plant matrix of cinnamon does not play a major role for bioavailability of coumarin, and the TDI of 0.1 mg/kg body weight daily can be used for risk assessment of coumarin exposure from cinnamon-containing meals.

In the case of coumarin from cinnamon tea, the extent of absorption may even be higher than that of isolated coumarin, and due to different galenics (fluid matrix); peak levels may reach distinctly higher values compared with isolated coumarin in capsule. Therefore, coumarin in cinnamon tea may even pose a higher risk compared with coumarin in tablets or cinnamon-containing meals (in case of exposure above the TDI) if the peak level above a threshold and not the AUC is the driving force for hepatotoxicity; this question is currently open.

The authors thank Ines Schirrmann for excellent technical assistance, as well as Heiko Ferber and Stefanie Thiel for additional measurements. The authors also thank Dr. Christian Steffen (Federal Institute for Drugs and Medical Devices, Germany) for support in clarifying the status of the study (food study, no clinical trial) and Dr. Hans Mielke for critical discussions. Special thanks go to all colleagues who participated in the study for their outstanding cooperation.

The authors have declared no conflict of interest.

6 References

[1] Egan, D., O'Kennedy, R., Moran, E., Cox, D. *et al.*, The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. *Drug Metab. Rev.* 1990, *22*, 503–529.

[2] Lake, B. G., Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. *Food Chem. Toxicol.* 1999, *37*, 423–453.

[3] EFSA, Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contacts with food (AFC) on a request from the commission related to coumarin. *EFSA J.* 2004, *104*, 1–36.

[4] BfR, Consumers, who eat a lot of cinnamon, currently have an overly high exposure to coumarin. Health Assessment No. 043/2006, 2006.

[5] Abraham, K., Wöhrlin, F., Lindtner, O., Heinemeyer, G., Lampen, A., Toxicology and risk assessment of coumarin: focus on human data. *Mol. Nutr. Food Res.* 2010, *54*, 228–239.

[6] Rychlik, M., Quantification of free coumarin and its liberation from glucosylated precursors by stable isotope dilution assays based on liquid chromatography-tandem mass spectrometric detection. *J. Agric. Food Chem.* 2008, *56*, 796–801.

[7] Sproll, C., Ruge, W., Andlauer, C., Godelmann, R., Lachenmeier, D. W., HPLC analysis and safety assessment of coumarin in foods. *Food Chem.* 2008, *109*, 462–469.

[8] Wöhrlin, F., Fry, H., Abraham, K., Preiss-Weigert, A., Quantification of flavoring constituents in cinnamon: high variation of coumarin in cassia bark from the German retail market and in authentic samples from Indonesia. *J. Agric. Food Chem.* 2010, *58*, 10568–10575.

[9] Miller, K. G., Poole, C. F., Chichila, T. M. P., Solvent-assisted supercritical fluid extraction for the isolation of semivolatile flavor compounds from cinnamons of commerce and their separation by series-coupled column gas chromatography. *J. High. Resol. Chromatogr.* 1995, *18*, 461–471.

[10] Abraham, K., Zimt und Cumarin: eine Klarstellung aus wissenschaftlich-behördlicher Sicht. *Dtsch. Lebensmittel-Rundschau* 2007, *103*, 480–487.

[11] SKLM (Senatskommission zur Beurteilung der gesundheitlichen Unbedenklichkeit von Lebensmitteln), natural constituents of foods: assessing the toxicity of substances administered as pure compounds compared to their ingestion as inherent food components. Statement of the DFG-Senate commission on Food Safety. 2006.

[12] EFSA Scientific Committee. Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements. *EFSA J.* 2009, *7*, 1249.

[13] Loprinzi, C. L., Kugler, J. W., Sloan, J. A., Rooke, T. W. *et al.*, Lack of effect of coumarin in women with lymphedema after treatment for breast cancer. *N. Engl. J. Med.* 1999, *340*, 346–350.

[14] Schmeck-Lindenau, H. J., Naser-Hijazi, B., Becker, E. W., Henneicke-von Zepelin, H. H., Schnitker, J., Safety aspects of a coumarin-troxerutin combination regarding liver function in a double-blind placebo-controlled study. *Int. J. Clin. Pharmacol. Ther.* 2003, *41*, 193–199.

[15] EMA (European Medicines Agency). Guideline on the investigation of bioequivalence. 2010.

[16] Ritschel, W. A., Brady, M. E., Tan, H. S., Hoffmann, K. A. *et al.*, Pharmacokinetics of coumarin and its 7-hydroxy-metabolites upon intravenous and peroral administration of coumarin in man. *Eur. J. Clin. Pharmacol.* 1977, *12*, 457–461.

[17] Ritschel, W. A., Brady, M. E., Tan, H. S., First-pass effect of coumarin in man. *Int. J. Clin. Pharmacol. Biopharm.* 1979, *17*, 99–103.

[18] Pelkonen, O., Rautio, A., Raunio, H., Pasanen, M., CYP2A6: a human coumarin 7-hydroxylase. *Toxicology* 2000, *144*, 139–147.

[19] Rautio, A., Kraul, H., Kojo, A., Salmela, E., Pelkonen, O., Interindividual variability of coumarin 7-hydroxylation in healthy volunteers. *Pharmacogenetics* 1992, *2*, 227–233.

[20] Merkel, U., Sigusch, H., Hoffmann, A., Grapefruit juice inhibits 7-hydroxylation of coumarin in healthy volunteers. *Eur. J. Clin. Pharmacol.* 1994, *46*, 175–177.

[21] EFSA, scientific opinion of the panel on food additives, flavourings, processing aids and materials in contact with food on a request from the European Commission on coumarin in flavourings and other food ingredients with flavouring properties. *EFSA J.* 2008, *793*, 1–15.

[22] Egan, D. A., O'Kennedy, R., Rapid and sensitive determination of coumarin and 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma by high-performance liquid chromatography. *J Chromatogr.* 1992, *582*, 137–143.

[23] Karalis, V., Macheras, P., Van Peer, A., Shah, V. P., Bioavailability and bioequivalence: focus on physiological factors and variability. *Pharm. Res.* 2008, *25*, 1956–1962.

[24] Yourick, J. J., Bronaugh, R. L., Percutaneous absorption and metabolism of coumarin in human and rat skin. *J. Appl. Toxicol.* 1997, *17*, 153–158.

[25] Rauma, A. L., Rautio, A., Pasanen, M., Pelkonen, O. *et al.*, Coumarin 7-hydroxylation in long-term adherents of a strict uncooked vegan diet. *Eur. J. Clin. Pharmacol.* 1996, *50*, 133–137.

[26] Shilling, W. H., Crampton, R. F., Longland, R. C., Metabolism of coumarin in man. *Nature* 1969, *221*, 664–665.

[27] Meineke, I., Desel, H., Kahl, R., Kahl, G. F., Gundert-Remy, U., Determination of 2-hydroxyphenylacetic acid (2HPAA) in urine after oral and parenteral administration of coumarin by gas-liquid chromatography with flame-ionization detection. *J. Pharm. Biomed. Anal.* 1998, *17*, 487–492.

[28] Mahavorasirikul, W., Tassaneeyakul, W., Satarug, S., Reungweerayut, R. *et al.*, CYP2A6 genotypes and coumarin-oxidation phenotypes in a Thai population and their relationship to tobacco smoking. *Eur. J. Clin. Pharmacol.* 2009, *65*, 377–384.

