

**Coumarin in flavourings¹
and other food ingredients with flavouring properties**

**Scientific Opinion of the Panel on Food Additives, Flavourings,
Processing Aids and Materials in Contact with Food (AFC)**

(Question No EFSA-Q-2008-677)

Adopted on 8 July 2008

PANEL MEMBERS

Fernando Aguilar, Herman Nybro Autrup, Susan Barlow, Laurence Castle, Riccardo Crebelli, Wolfgang Dekant, Karl-Heinz Engel, Nathalie Gontard, David Michael Gott, Sandro Grilli, Rainer Gürtler, John Christian Larsen, Catherine Leclercq, Jean-Charles Leblanc, F. Xavier Malcata, Wim Mennes, Maria Rosaria Milana, Iona Pratt, Ivonne Magdalena Catharina Maria Rietjens, Paul P. Tobback, Fidel Toldrá.

SUMMARY

The Panel considered the toxicity studies and the studies on the metabolism of coumarin in humans with CYP2A6 polymorphism that have become available since the last opinion of 2004, as well as clinical studies, and concluded to maintain the TDI of 0.1 mg coumarin/kg bw allocated in the 2004 opinion.

Considering the toxicity data on coumarin, including the timing of the onset of liver effects, recovery of these effects after cessation of exposure to coumarin and the elimination half-life, the Panel concluded that exposure to coumarin resulting in an intake 3 times higher than the TDI for one to two weeks is not of safety concern.

Key words: coumarin, safety assessment, CYP2A6, CYP2AA6*

¹ For citation purposes: 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. *The EFSA Journal (2008) 793, 1-15.*

BACKGROUND

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (the Panel) of the European Food Safety Authority (EFSA) adopted in 2004 an opinion in which it was concluded that coumarin was not genotoxic in experimental animals and therefore a Tolerable Daily Intake (TDI) could be allocated. The Panel also considered that the majority of the human population have a major pathway for the metabolism of coumarin (the 7-hydroxycoumarin pathway) that differs from that in the rat (the 3,4-coumarin epoxidation pathway) in which a reactive epoxide is formed. This 3,4-coumarin epoxide route has been linked to the hepatotoxicity and the carcinogenic effects of coumarin observed in long-term studies in rats and mice. The Panel (EFSA, 2004) noted that comparative studies in humans from South Europe and Asia show that a considerable number of individuals exhibit genetic polymorphism in that they have a considerable reduction in the capacity of the 7-hydroxycoumarin pathway. However, it is not known to what extent this reduction may trigger the involvement of other pathways in the metabolism of coumarin. The Panel (EFSA, 2004) therefore concluded that the hepatotoxic responses should be taken into account in setting a TDI and that in applying safety factors to the No-Observed-Adverse-Effect Level (NOAEL) for hepatotoxicity, it would be prudent to use a factor for potential interspecies variation, together with a factor of 10 for potential individual differences between humans. The overall NOAEL for liver toxicity in the most sensitive animal species, based on hepatotoxicity in a two year dog study, was 10 mg coumarin/kg body weight (bw)/day. Applying a safety factor of 100, the Panel established a TDI of 0 - 0.1 mg coumarin/kg bw.

New *in vitro* studies on the metabolism of coumarin in humans and possible matrix effects of cinnamon (which contains coumarin) on the uptake and metabolism of coumarin as well as human toxicity data from the use of coumarin as a drug have now become available. In addition, two new risk assessments of coumarin have been performed by Felter *et al.* (2006) and the German Federal Institute for Risk Assessment (BfR) (2006a, 2006b).

ACKNOWLEDGEMENT

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food wishes to thank Gerrit Speijers for his contribution to the draft Opinion.

TERMS OF REFERENCE

The Commission requests the European Food Safety Authority to:

- 1) Consider if the Tolerable Daily Intake (TDI) for coumarin set in the Opinion of the Scientific Panel on Food Additives, flavourings, Processing Aids and Materials in Contact with Food (AFC Panel) of October 2004 is still valid, taking into account the additional information provided.
- 2) Consider the possible consequences on the health of the consumers when the TDI would be slightly exceeded during a period of one or two weeks.

ASSESSMENT

Previous assessments of coumarin

As reported in the previous Opinions in 1994 and 1999 by the Scientific Committee for Food (SCF), coumarin is a well known hepatotoxic compound in the rat whereas in mice the primary effect is lung injury (SCF, 1997, 1999). Long-term studies showed induction of liver and lung tumours at high doses in rats and mice, respectively, usually associated with hepatic and pulmonary toxicity. The data on the genotoxic potential of coumarin available at the time of the previous SCF opinions did not allow the exclusion with certainty of a genotoxic mechanism for the coumarin-mediated carcinogenicity in experimental animals. For that reason the SCF requested studies to examine the potential of coumarin to induce DNA-adduct formation in the liver and the kidney after administration of [¹⁴C]-ring-labelled coumarin to rats, to resolve the issue of the genotoxic potential of coumarin. These and some additional *in vivo* studies relevant to the toxicity of coumarin became available to EFSA (2004). From the studies on adduct formation it was concluded that coumarin does not bind covalently to DNA in target organs. This result was further supported by another study in which coumarin did not cause Unscheduled DNA Synthesis (UDS) in hepatocytes of male Sprague-Dawley (SD) rats *in vivo* after administration of coumarin at dose levels up to the Maximum Tolerated Dose (MTD), as well as by a negative micronucleus assay in mice.

Comparative studies on the toxicokinetics and metabolism of coumarin in humans and rodents have confirmed species differences, as reported by the SCF (1999). 7-Hydroxycoumarin formation, mediated by the enzyme CYP2A6, is the major detoxifying pathway in humans, whereas in rats and mice bio-activation of coumarin to 3,4-coumarin epoxide is the prevalent biotransformation reaction. 3,4-Coumarin epoxide is rapidly converted to *o*-hydroxyphenyl acetaldehyde (*o*-HPA) in rodents and some other animal species. In rats the detoxification process of *o*-HPA is slow compared to other animal species, explaining the fact that in long-term studies liver toxicity and hepatic tumours were only observed in rats (Lake, 1999). On the other hand, 3,4-coumarin epoxide formation in mouse lung Clara cells and the relatively higher abundance of these cells in the terminal bronchiolar region have been reported as the major determinants for the susceptibility of mice to lung toxicity and carcinogenicity. These lung effects are also dependent on the mode of administration (gavage or dietary) and are species-specific with limited relevance to human health (Lake, 1999).

Since the new studies showed that coumarin is not an *in vivo* genotoxic agent, a threshold-based approach was considered justified by the Panel in 2004. A NOAEL for toxicity to target tissues in the long-term oral studies previously reported and evaluated (see Annex 1 for further details) was used to derive a TDI for coumarin. From these data it was concluded that the overall NOAEL was 10 mg coumarin/kg bw/day, based on the hepatotoxicity in dogs seen at the next higher dose level (25 mg coumarin/kg bw/day). Applying a safety factor of 100, the Panel established a TDI of 0 - 0.1 mg coumarin/kg bw (EFSA, 2004).

TOXICITY AND METABOLISM STUDIES OF COUMARIN IN ANIMALS OR HUMANS RELEVANT FOR RISK ASSESSMENT, PUBLISHED OR REPORTED AFTER 2004, OR STUDIES WHICH HAVE BEEN RECONSIDERED

Metabolism and toxicokinetic studies

Burian *et al.* (2003) concluded that there is no evidence that the polymorphism in CYP2A6*² is a determinant of coumarin-associated liver dysfunction in humans. In contrast, Farinola and Piller (2007) speculated that a reduction in 7-hydroxylation will lead to shunting of metabolism into other pathways for coumarin.

Vassallo *et al.* (2004) studied the metabolic detoxification of coumarin and species differences in coumarin-induced hepatotoxicity. This study was already included in the EFSA (2004) evaluation and Opinion on coumarin. However, these investigators did not study the potential shift of the metabolic pathways in humans with polymorphisms in which 7-hydroxycoumarin formation is blocked, and whether this might be linked to hepatotoxicity in these humans.

Lewis *et al.* (2006) reviewed studies on the metabolism catalysed by human P450 enzymes published before 2004, which thus have been considered by EFSA (2004). They referred to kinetic studies by Zhuo *et al.* (1999) and Born *et al.* (2002), who reported that CYP1A1, CYP1A2, CYP2B6, CYP2E1 and CYP3A4 could all catalyse the metabolism of coumarin to the 3,4-coumarin epoxide pathway, reflected in the appearance of *o*-HPA in urine, whereas CYP2A6 catalyses exclusively the formation of 7-hydroxycoumarin.

Peamkrasatam *et al.* (2006) evaluated the *in vivo* metabolism of coumarin and nicotine to assess genetic polymorphism of CYP2A6 in a Thai population. However, they did not study the metabolic pathway in subjects deficient in the 7-hydroxycoumarin pathway. Similarly, Aoki *et al.* (2006) who characterised the humanised liver from chimeric mice using coumarin, to prepare a human CYP2A6 and mouse CYP2A5 probe, did not study which pathway for coumarin metabolism will increase in case of polymorphism. Satarug *et al.* (2006) only studied genetic and environmental influences on therapeutic and toxicity outcomes with CYP2A6 (for more details see Annex 1)

In recent Physiologically-Based BioKinetic (PBBK) model studies by Rietjens *et al.* (2007, 2008) using results obtained with liver microsomes from rats and from two different human donors, having respectively a high and a low 7-hydroxylation activity, it was shown that the *o*-HPA and *ortho*-phenyl acetic acid (*o*-HPAA) metabolites were slowly formed in human microsomes with high 7-hydroxylation capacity compared with the rat microsomes. The metabolites *o*-HPA and *o*-HPAA are formed from 3,4-coumarin epoxide, which is highly reactive and has a very short half-life (20 seconds) when synthesised. 3,4-Coumarin epoxide and the further metabolites, *o*-HPA and *o*-HPAA, are not formed in the 7-hydroxycoumarin pathway. The *V*_{max} and *K*_m values obtained for the different pathways were used in the PBBK model. The polymorphic allele does not code for the active enzyme in the 7-

² CYP2A6*: In the literature often used to indicate that this microsomal enzyme is deficient in catalysing the 7-hydroxylation of coumarin

hydroxycoumarin pathway (CYP2A6*). Thus, in individuals heterozygous for the activity of this enzyme the activity is decreased, and in homozygous individuals the pathway is strongly impaired. By setting the 7-hydroxycoumarin pathway at zero, the PBBK model mimics the *in vivo* situation of a homozygous individual.

The model predicts that the *rate* of the 3,4-coumarin epoxide pathway in humans deficient in 7-hydroxylation activity, as reflected in the C_{max} for *o*-HPA formation in the liver, would be increased 70-fold compared to humans without the polymorphism. However, for the *Area Under the Curve* (AUC) of *o*-HPA formation (reflecting the total amount of *o*-HPA formed), the change would be more pronounced in that the AUC would be increased 500-fold in the deficient individuals. The C_{max} value for the deficient human subject was an order of magnitude lower than for rats, whereas for the wild-type subject it was 3 orders of magnitude lower than in rats.

In summary, there are no *in vivo* studies available on the metabolic pathway of coumarin in humans that have homozygous alleles for polymorphism in the enzyme CYP2A6* which may be impaired and in which the metabolism of coumarin to 7-hydroxycoumarin is likely to be strongly reduced.

Clinical data on hepatotoxicity of coumarin in the human population

Since the early seventies of the last century, coumarin has been used as a medicinal drug for the treatment of different diseases (e.g. oedema, cancer, infections, chronic fatigue syndrome). For the treatment of lymph oedema or other protein oedemas, coumarin is also used in combination with troxerutin.

Taking all the data from clinical experience with coumarin together (see Annex 2 for further details), there is much evidence for a human subpopulation, with a percentage frequency in the one-digit range, which is more susceptible than the animal species investigated. As only people of this subgroup would be susceptible to hepatotoxicity from coumarin, the clinical studies do not provide evidence for a dose-dependency regarding the frequency of toxicity in this subgroup, but do provide information on dose-dependency regarding the severity of hepatotoxicity in susceptible individuals. Evaluating the available case reports on hepatotoxicity with known doses of coumarin (51 cases from different countries), Bergmann (1999) identified a daily dose of 25 mg to be the lowest documented dose capable of inducing this response (possibly, no lower doses were applied to patients).

The liver toxicity observed after oral administration in humans is of relevance, and therefore should be used in the safety assessment of coumarin. Whether exposure to pulse doses by giving tablets or comparable forms may cause stronger hepatotoxic effects in humans than coumarin consumed via food is unknown. As coumarin in relatively high amounts is often consumed as particular (traditional) foods, the pulse exposure in the medical situation might be representative for these foods.

Exposure estimates reported since 2004

The EFSA opinion of 2004 also included a risk characterisation. The TDI of 0.1 mg coumarin/kg bw would be equivalent to 6 mg for a person of 60 kg. The Panel calculated a Theoretical Added Maximum Daily Intake (TAMDI) of 1.3 - 1.5 mg coumarin/day, which, at

a body weight of 60 kg, would be equivalent to 0.02 mg coumarin/kg bw/day. These intake scenarios were below the TDI (EFSA, 2004).

The German Federal Institute for Risk Assessment (BfR, 2006a,b) reported exposure assessments for coumarin taking into account oral and dermal routes of exposure. They arrived at a total daily exposure of 0.19 mg + 0.08 mg = 0.27 mg/kg bw for 2 to 5 year-old children, the group with the highest exposure, which would result from the worst case considerations of oral and dermal coumarin exposure. At least in Germany, mainly cassia cinnamon is used as flavouring ingredient that contains relatively high concentrations of coumarin. Very recently Sproll *et al.* (2008) found appreciable amounts of coumarin in bakery products and breakfast cereals (mean 9 mg/kg), with the highest concentrations up to 88 mg/kg in certain cookies flavoured with cinnamon. The BfR also identified a specific exposure situation of concern that can arise from the consumption of large amounts of cinnamon powder in the form of capsules which are sold as food supplements or as dietetic foods to reduce blood sugar in persons with type II diabetes mellitus.

Hazard and risk assessments published since 2004

Felter *et al.* (2006) published a safety assessment of coumarin using the species-specificity in the toxicokinetics. They stated that on the basis of the difference in metabolism between human and the rat, the safety factor applied to establish an Acceptable Daily Intake (ADI) should be a factor 4 lower than the usual factor of 100, as applied by EFSA (2004). However, the Panel considered that this was not supported by study results. All references cited by Felter *et al.* (2006) date from 2004 or before and no new data were brought forward since the EFSA Opinion of 2004. They did not consider what would happen in cases of low activity of CYP2A6. There is no adequate evidence to exclude that other pathways lead to *o*-HPA and *o*-HPAA via the 3,4-coumarin epoxide pathway.

Rather than using the NOAEL from a dog study, which was the most sensitive species tested (EFSA, 2004), Felter *et al.* (2006) used the NOAEL obtained from a rat study in their assessment. However, as in the dog, the metabolism of coumarin in the rat is different from that in humans. From a metabolism point of view it might be more realistic to use results from the baboon, which more closely resembles “normal” humans in its metabolism of coumarin. This has been shown in two studies (Gangolli *et al.*, 1974; Waller and Chasseaud, 1981). Baboons are extensive 7-hydroxylators of coumarin with 60-66 % of the dose being rapidly excreted in the urine as 7-hydroxycoumarin. However, as CYP2A6*-deficient humans also have to be considered, the evaluation cannot be based only on animal species in which the metabolism of coumarin is similar to that in wild-type humans.

In 2006 the BfR (2006a,b) discussed the risk and exposure assessments on coumarin performed by different national and international bodies such as the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) and the EFSA (2004). They also took into account the safety assessments based on the use of coumarin as a medicinal drug with respect to hepatotoxic effects in humans. The BfR stated that “regarding the quantitative evaluation of the human studies, the lowest daily dose of coumarin that produced liver toxicity can be used as a point of departure for the risk assessment”. Based on the data of Bergmann (1999), who identified a daily dose of 25 mg to be the lowest documented dose capable of inducing this hepatotoxic response, the BfR applied an extrapolation factor of 5 (assuming a typical slope of the dose-response curve) resulting in a level of 5 mg coumarin per day, which is expected to cause no adverse effects, even in sensitive people. This corresponds to a TDI of 0.1 mg/kg

bw. Thus, the TDI of 0.1 mg/kg bw established by EFSA in 2004 based on animal data was further supported by the evaluation of the BfR (2006a) based on human data.

Evaluation of the present data and discussions since 2004

On request of the Commission the AFC Panel considered new data and assessments on the metabolism and toxicity of coumarin and revisited the assessment of the TDI performed in 2004.

In the 2004 Opinion on coumarin it was explained that the Panel did not have adequate data on the metabolism of coumarin in humans who were low in activity of CYP2A6, the 7-hydroxylating enzyme (EFSA, 2004). The 7-hydroxylation pathway was considered of less toxicological concern than the pathway leading to 3,4-coumarin epoxide (which is further converted to *o*-HPA and *o*-HPAA), which is the major pathway in rats and some other animal species.

However, for humans, in particular those who are homozygous for the low activity form of CYP2A6*, no new data on the other pathways have become available. Only two reports on PBBK-model studies (Rietjens *et al.*, 2007, 2008) are available in which the different metabolic pathways were simulated on the basis of observations from human liver microsome samples with different enzyme activity. The PBBK model (Rietjens *et al.*, 2008) suggests that simulating complete lack of activity of CYP2A6 would increase the exposure of humans to the toxic coumarin metabolite by 70-fold based on C_{max}, and by 500-fold based on the AUC. The model has not been validated in humans and the studies are not yet published. However, it currently provides the best available information to estimate the consequence of a complete lack of activity of CYP2A6 activity for coumarin metabolism in humans. Furthermore, at present it cannot be excluded that mechanisms other than CYP2A6 polymorphism play a role in the toxicity of coumarin in humans.

In rats, coumarin has been found to be carcinogenic in the liver. The carcinogenicity is related to cytotoxicity caused by reactive coumarin metabolites, generated via the 3,4-coumarin epoxide route. As this metabolic route might be the preferred route in persons with impaired CYP2A6 activity, such people might be at higher risk for coumarin carcinogenicity than those people with normal CYP2A6 activity. However, as it has been demonstrated that the carcinogenicity of coumarin is not linked to genotoxicity (EFSA, 2004) it remains valid to assess the safety of coumarin based on a threshold approach, even for the poorly metabolising subpopulation. As with the wild-type population with normal metabolising activity, an increased risk of carcinogenicity is not expected in the subpopulation with impaired CYP2A6 activity at levels of exposure which do not lead to irreversible toxicity in the liver.

Another point of concern are the human data collected from the medicinal use of coumarin, which were evaluated by the BfR. Bergmann (1999) analysed the dose-response for the incidence of liver toxicity based on data from German, Irish and French reports. This analysis identified a daily dose of 25 mg per person to be the lowest dose documented capable of inducing an hepatotoxic response. The BfR applied a factor of 5 to extrapolate from the LOAEL to a NOAEL of 5 mg daily. In this manner, the BfR (2006a) derived a TDI of 0.1 mg coumarin/kg bw. This figure is not in conflict with the TDI of 0.1 mg coumarin/kg bw established by EFSA in 2004.

Thus, the evaluation of the data, discussion papers, and assessments published since 2004 does not provide a basis for changing the TDI for coumarin of 0.1 mg/kg bw.

The Panel was also requested to consider the possible consequences on the health of consumers when the TDI would be slightly exceeded during a period of one or two weeks. The Panel noted that a conservative assessment of exposure leads to an intake of coumarin of about a 3 times higher than the TDI for coumarin. The Panel considers that an ADI or TDI should, in principle, not be exceeded. However, it is widely agreed that a slight exceeding of the intake of a compound above its ADI for a short period (in relation to the duration of onset of the critical effect) would not be of health concern provided that the ADI is not exceeded on a long-term basis (Barlow *et al.*, 1999). The magnitude and length of any acceptable exceeding of an ADI is dependent on the toxicological properties of the compound in question, and should always be evaluated on a case-by-case basis. For coumarin, the Panel noted that the onset of effects on the liver, as indicated by slight increases in liver enzyme activities in serum, as a sub-acute effect in humans at the lower doses used in clinical studies of coumarin, can appear after one or two weeks of daily exposure (Bergmann, 1999), and that the effects reversed after cessation of the exposure. Therefore, the Panel would not have safety concerns about a 3-fold exceeding of the TDI for coumarin for a period of one to two weeks.

CONCLUSIONS

The Panel considered the toxicity studies and the studies on the metabolism of coumarin in humans with CYP2A6 polymorphism that have become available since the last opinion of 2004, as well as clinical studies, and concluded to maintain the TDI of 0.1 mg coumarin/kg bw allocated in the 2004 Opinion.

Considering the toxicity data on coumarin, including the timing of the onset of liver effects, recovery of these effects after cessation of exposure to coumarin and the elimination half-life, the Panel concluded that exposure to coumarin resulting in an intake 3 times higher than the TDI for one to two weeks is not of safety concern.

REFERENCES

- Andrejak, M., Gersberg, M., Sgro, C., Decocq, G., Hamel, J.G., Monin, M., and Gras, V., 1998. French pharmacovigilance survey evaluating the hepatic toxicity of coumarin. *Pharmacoepidemiol. Drug Saf.*, **7**, 45-50.
- Adam, B.S., Pentz, R., Siegers, C.P., Strubelt, O., and Tegtmeier, M., 2005. Troxerutin protects the isolated perfused rat liver from a possible lipid peroxidation by coumarin. *Phytomedicine*, **12**, 52-61.
- Aoki, K., Kashiwagura, Y., Horie, T., Sato, H., Tateno, C., Ozawa, N. and Yoshizato, K., 2006. Characterisation of humanized liver from chimeric mice using coumarin as a human CYP2A6 and mouse CYP2A5 probe. *Drug. Metab. Pharmacokinet.* **21**, 277-285.
- Barlow, S., Pascal, G., Larsen, J.C. and Richold, M., 1999. ILSI Europe Workshop on the significance of excursions of Intake above the Acceptable Daily Intake (ADI), *Regulatory Toxicology and Pharmacology*, **30**, S3-S121.
- Basset, M.L. and Dahlström, J.E., 1995. Liver Failure while taking coumarin. *Med. J. Australia*, **163**, 106.
- Beinssen, A.P.C., 1994. Possible coumarin hepatotoxicity. *Med. J. Australia*, **161**, 725.
- Bergmann, K. von, 1999. Sachverständigengutachten zur Beurteilung von Cumarin in Arzneimittel in Bezug auf lebertoxische Wirkung beim Menschen. Dezember 21 1999 Bonn, pg 1-23. Rheinische Freidrich-Wilhelms-Universität Bonn, Deutschland. Available from the Federal Institute for Risk Assessment (BfR), Berlin
- BfR(Federal Institute for Risk Assessment), 2006a. Consumers, who eat a lot of cinnamon, currently have an overly high exposure to coumarin. BfR Health Assessment No. 043/2006, 16 June 2006.
- BfR(Federal Institute for Risk Assessment), 2006b. High daily intakes of cinnamon: Health risk cannot be ruled out. BfR Health Assessment No 44/2006, 18 August 2006.
- Born, S.L., Caudill, D., Fliter, K.L. and Purdon M.P., 2002. Identification of the cytochromes P450 that catalyze coumarin 3,4-epoxidation and 3-hydroxylation. *Drug Metabolism and Disposition*, **30**, 483-487.
- Burian, M., Freudenstein, J., Tegtmeier, M., Naser-Hijazi, B, Henneicke-von Zepelin, H.H. and Legrum, W., 2003. Single copy of variant CYP2A6 alleles does not confer susceptibility to liver dysfunction in patients treated with coumarin. *International Journal of Clinical Pharmacology and Therapeutics*, **41**, 141-147.
- Casley-Smith, J.R. and Casley-Smith, J.R., 1995. Frequency of coumarin hepatotoxicity. *The Medical J. Australia*, **162**, 391
- Cox, D., O'Kennedy, R. and Thornes, R.D., 1989. The rarity of liver toxicity in patients treated with coumarin (1,2-benzopyrene). *Human Toxicol.* **8**, 501-506.

EFSA (European Food Safety Authority), 2004. 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. The EFSA Journal, **104**, 1-36.

Evans, J.G., Gaunt, I.F. and Lake, B.G., 1979. Two-year toxicity study on coumarin in the baboon. Food. Cosmet. Toxicol., **17**, 187-193.

Farinola, N., and Piller, N.B., 2007. CYP2A6 polymorphism: is there a role for pharmacogenomics in preventing coumarin-induced hepatotoxicity in lymphedema patients? Pharmacogenomics, **8**, 1462-2416.

Felter, S.P., Vassallo, J.D., Carlton, B.D. and Daston, G.P., 2006. A safety assessment of coumarin into account species-specificity of toxicokinetics. Food and Chemical Toxicology, **44**, 462-475.

Gangolli, S.D., Shilling, W.H., Grasso, P. and Gaunt, I.F. 1974. Studies on the metabolism and hepatotoxicity of coumarin in the baboon. Biochemical Society Transactions, 54th meeting, **2**, 310-312.

Haddidi, H., Zahlsen, K., Idle, J.R. and Cholerton, S., 1997. A single amino acid substitution (Leu160His) in cytochrome P450 CYP2A6 causes switching from 7- hydroxylation to 3-hydroxylation of coumarin. Food and Chemical Toxicology, **35**, 903 –907.

Koch, S., Beurton, I., Bresson-Hadni, S., Monnot, B., Hrusovsky, S., Becker, M., VanLemmens, C., Carbillet, J.P., and Miquet, J.P., 1997. Acute cytolytic hepatitis caused by coumarin. Two cases. Gastroenterology and Clinical Biology, **21**, 223-225.

Lake, B.G., 1999. Coumarin metabolism, toxicity and carcinogenicity: relevance for human risk assessment. Food and Chemical Toxicology, **37**, 423-453.

Lewis, D.F.V., Ito, Y., Lake, B.G., 2006. Metabolism of coumarin by human P450s: A molecular modelling study. Toxicology *in Vitro*, **20**, 256-264.

Loprinzi, C.L., Kugler, J.W., Sloan, J.A., Rooke, T.W., Quella, S.K., Novotny, P., Mowat, R.B., Michalak, J.C., Stella, P.J., Levitt, R., Tschetter, L.K., Windschitl, H., 1999. Lack of effect of coumarin in women with lymphedema after treatment for breast cancer. N Engl J Med. 340(5):346-50.

Morrison, L. and Welsby, P.D., 1995. Side-effects of coumarin. Postgrad. Med. J., **71**, 701 – 702.

Peamkrasatam, S., Sriwatanakul, K., Kryotani, K., Fujieda, M., Yamazaki, H., Kamataki, T. and Yoovathaworn, K., 2006. *In vivo* evaluation of coumarin and nicotine as Probe drug to predict the metabolic capacity of CYP2A6 due to genetic polymorphism in Thais. Drug Metab. Pharmacokinet., **21**, 475-484.

Rietjens, I.C.M., Zaleska, M., Boersma, M.G. and Punt, A., 2007. Species differences in coumarin metabolism. Physiology based biokinetic (PBBK) study on the bioactivation of coumarin to o-HPA in the liver of CYP2A6 deficient and CYP2A6 wild-type human subjects as compared to o-HPA formation in rat liver. Report of division of Toxicology, Wageningen, NL.

Rietjens, I.C.M., Boersma, M.G. and Punt, A., 2008. Matrix effects of cinnamon on coumarin metabolism and PBBK modelling of o-HPA formation in the liver. Report of division of Toxicology, Wageningen, NL.

Rietjens, I.C.M., Zaleska, M., Boersma, M.G. and Punt, A. Differences in simulated liver concentrations of toxic coumarin metabolites in rats and different human populations evaluated through physiologically based biokinetic (PBBK) modeling. *Toxicology in vitro* (2008) (in press).

Satarug, S., Tassaneeyakul, W., Na-Bangchang, K., Cashman, J.R. and Moore, M.R., 2006. Genetic and Environmental Influences on therapeutic and toxicity outcomes: Studies with CYP2A6. *Current Clinical Pharmacology*, **1**, 291-309.

SCF(Scientific Committee for Food), 1997. Opinion on coumarin (a constituent of natural flavouring source material limited by annex II of flavouring directive 88/388/EEC) expressed on 16 December 1994. Reports of the Scientific Committee for Food, Thirty-sixth Series, pp 13-20, European Communities, 1997.

SCF(Scientific Committee for Food), 1999. Opinion on coumarin (a constituent of natural flavouring source material limited by annex II of flavouring directive 88/388/EEC) expressed on 22 September 1999. Reports of the Scientific Committee for Food, European Commission, Health & Consumer Protection, Directorate-General. SCF/CS/ADD/FLAV/61 final 29/9/99, http://www.europa.eu.int/comm/dg24/health/sc/scf/index_en.html .

Schmeck-Lindenau, H.J., Naser-Hijazi, B., Becker, E.W., Hennicke-von Zepelin, H.H. and Schniker, J., 2003. *International Journal of Clinical Pharmacology and Therapeutics*, **41**, 193-199.

Sproll, C., Ruger, W., Andlauer, C., Godelmann, R. and Lachenmaier, D.W., 2008. HPLC analysis and safety assessment of coumarin in foods. *Food Chemistry*, **109**, 462-469.

Vanscheidt, W., Rabe, E., Naser-Hijazi, B., Ramelet, A.A., Partsch, H., Diehm, C., Schultz-Ehrenburg, U., Spengel, F., Wirsching, M., Goetz, V., Schnitker, J. and Henneicke-von Zepelin, H.H., 2002. The Efficacy and safety of a coumarin-/troxerutin-combination (SB-LOT) in patients with chronic venous insufficiency: A double blind placebo-controlled randomised study. *VASA*, **31**, 185-190.

Vassallo, J.D., Hicks, S.M., Daston, G.P. and Lehman-McKeeman, L.D., 2004. Metabolic Detoxification Determines Species Differences in Coumarin-Induced Hepatotoxicity, *Toxicological Sciences* **80**, 249-257.

Waller, A.R. and Chasseaud, L.F., 1981. The metabolic fate of [¹⁴C]coumarin in baboons. *Food Cosmet. Toxicol.*, **19**, 1-6.

Zhuo, X., Gu, J., Zhang, QY., Spink, D.C., Kaminsky, L.S., Ding, X., 1999. Biotransformation of coumarin by rodent and human cytochromes P-450: metabolic basis of tissue-selective toxicity in olfactory mucosa of rats and mice. *J Pharmacol Exp Ther.* **288**, 463-471.

ANNEX 1

Toxicity data from 1994 SCF Opinion

Several sub-chronic and chronic studies with mice and rats have been performed. The rat strains studied were Fisher 344, Osborne-Mendel and Sprague-Dawley. In most cases coumarin was administered via the diet. In the dietary studies, the no-effect level for hepatotoxic effects ranged from doses equivalent to 50 to 130 mg coumarin/kg bw/day, whereas in mice the no-effect levels were higher. Other species, including gerbils and dogs, revealed hepatotoxic effects after dietary administration of 50 mg coumarin/kg bw/day or more. In the dog, oral doses of 25 mg coumarin/kg bw/day for more than 100 days caused histological liver damage, whereas 10 mg/kg bw/day was a NOAEL. As in the rat, urinary excretion of 7-hydroxycoumarin is low in dogs, hamsters and some strain of mice. Baboons, which have a coumarin metabolism very similar to humans (Gangolli *et al.*, 1974; Waller and Chasereaud, 1981) only showed slight liver effects; increased liver weights and dilatation of the endoplasmic reticulum, a finding consistent with early stages of intracellular oedema in hepatocytes in three of the four animals fed a dietary dose of 67.5 mg coumarin/kg bw/day for two years. No ultramorphological changes were observed at a dietary dose of 22.5 mg coumarin/kg bw and with conventional histopathological examination no changes were seen at the highest (67.5 mg coumarin/kg bw/day) dose level (Evans *et al.*, 1979). When coumarin was given by gavage to mice an increase in hepatocellular adenomas or combined adenomas and carcinomas was seen at dose levels of 50 and 100 mg/kg bw/day in females, but not at a dose level of 200 mg coumarin/kg bw/day. No such effects were found in males. From these data it can be concluded that the overall NOAEL is 10 mg coumarin/kg bw/day, based on the hepatotoxicity in dogs seen at the next higher dose level (25 mg coumarin/kg bw/day).

In contrast to rodents and dogs, in humans the detoxifying pathway leading to 7-hydroxycoumarin formation is prevalent. However, a CYP2A6 polymorphism exists, particularly in Asia and Southern Europe, and people having this polymorphism show a decreased capacity to metabolise coumarin to 7-hydroxy coumarin. However, it is not known which pathway(s) become(s) more important in such individuals and whether coumarin may be metabolised to a larger extent to coumarin epoxide (CE) and o-hydroxyphenyl acetaldehyde (o-HPA). For this part of the human population the outcome of the rat and dog studies may be relevant. A study by Burian *et al.* (2003) suggested that the CYP2A6 polymorphism might be of very limited relevance for the determination of the population spread in sensitivity to coumarin. However, the basis for this conclusion is unclear because no homozygous individuals with both alleles defective were represented in the studied group, and it has been described that homozygosity for the defective allele has much greater impact on coumarin metabolism than heterozygosity (Hadidi, 1997). In addition, in the study by Burian *et al.* (2003) data on other pathways of coumarin metabolism were not collected. The EFSA (2004) therefore concluded that hepatotoxicity as observed in rodents and dogs should be taken into account in setting a TDI. In applying safety factors to the NOAEL for hepatotoxicity, it was considered prudent to use a factor of 10 for potential interspecies variation, together with a factor of 10 for potential individual differences between humans. The overall NOAEL for liver toxicity in the most sensitive animal species was based on a two year dog study and was 10 mg/kg bw/day. Applying a total safety factor of 100 to this overall NOAEL, it was concluded that a TDI of 0 – 0.1 mg coumarin/kg bw could be established. It was also concluded (EFSA, 2004) that studies aimed at establishing which alternative

metabolism pathway would be increased in the case of decreased 7-hydroxycoumarin formation in humans would be helpful. Indeed, if the alternative pathway did not lead to hepatotoxic metabolites as observed for the 3,4-epoxidation of coumarin, a lower interspecies safety factor could be justified and applied.

Peamkrasatam *et al.* (2006) evaluated the *in vivo* metabolism of coumarin and nicotine to assess genetic polymorphism of CYP2A6 in a Thai population. The frequency of CYP2A6 alleles found in this study was: CYP2A6*1A = 32 %, CYP2A6*1B= 27 %, CYP2A6*9 = 20 %, CYP2A6*4 = 14 %, CYP2A6*7 = 5 % and CYP2A6*10 = 2 %. Subjects having CYP2A6* 1A/1B were found to have a higher concentration of 7-hydroxycoumarin in the plasma compared with those with the other genotypes. In contrast, subjects with CYP2A6*4/7 and CYP2A6*7/7 had just detectable levels in urine, and CYP2A6*9 allele clearly resulted in reduced enzyme activities. Despite the absence of the homozygote for CYP2A6*10 allele, the presence of CYP2A6*10 allele significantly reduced the enzyme activities for the 7-hydroxycoumarin pathway. Unfortunately, Peamkrasatam *et al.* (2006) did not study the metabolic pathway used by subjects deficient in the 7-hydroxycoumarin pathway. Similarly, Aoki *et al.*, (2006) who characterised the humanised liver from chimeric mice using coumarin to prepare a human CYP2A6 and mouse CYP2A5 probe, did not study which pathway for coumarin metabolism will increase in case of polymorphism. Satarug *et al.*, (2006) only studied genetic and environmental influences on therapeutic and toxicity outcomes with CYP2A6.

ANNEX 2

Clinical data on hepatotoxicity of coumarin in the human population

Since the early seventies of the last century, coumarin has been used as a medicinal drug for the treatment of different diseases (e.g. oedema, cancer, infections, chronic fatigue syndrome). For the treatment of lymph oedema or other protein oedemas, coumarin is also used in combination with troxerutin. This rutoside derivative is known as a radical scavenger with antioxidant effects, and treatment with the flavanoid may increase the healing of capillary endothelial defects. Troxerutin is also claimed to protect the liver against oxidative damage by coumarin, as has been demonstrated in hepatocytes *in vitro* and in the isolated perfused rat liver (Adam *et al.*, 2005). As already mentioned in the SCF opinion (1999), hepatotoxicity has been reported in a number of patients treated with coumarin (Beinssen, 1994; Casley-Smith and Casley-Smith, 1995; Morrison and Welsby, 1995; Bassett and Dahlström, 1995; Koch *et al.*, 1997). The severity of this adverse drug reaction ranged from elevated liver enzymes in serum only to clinical hepatitis and liver failure leading to death of the patients in a few cases. This has caused the authorities to withdraw coumarin from the market in France (Andrejak *et al.*, 1998) and other countries.

The frequency of hepatotoxic responses in patients treated with coumarin can be estimated from clinical trials. Cox *et al.* (1989) treated 2173 patients with different diseases receiving daily doses of 25 mg (chronic infections) to 2000 mg coumarin (advanced renal cell carcinoma and glioma). The majority of patients received 100 mg coumarin daily for one month, followed by 50 mg daily for two years. Hepatotoxic effects including elevated liver enzymes in serum were reported in 8 patients (0.37 %). For this calculation, five other patients were not considered who developed elevated enzyme levels which returned to normal while still on coumarin. For the assessment of the frequency of hepatotoxic responses in the human population, the reliability of this trial is limited, as different groups of patients were treated with different doses without inclusion of a placebo group. Furthermore, blood was collected every 3 months only. More significant data on the frequency of hepatotoxicity can be obtained from systematic studies with randomised and placebo-controlled design. Two of such studies are available for coumarin:

In the study of Loprinzi *et al.* (1999), 140 women with chronic lymph oedema received 200 mg of oral coumarin or placebo twice daily for six months and then the other treatment for the following six months (cross over design). Serologic evidence of liver toxicity was observed in 9 women (6 % of the study group), including one woman who developed jaundice. In the placebo period, no elevated liver enzymes in serum were recorded. The authors were unable to identify any predisposing factors, such as co-therapy with tamoxifen, an inducer of CYP3A4 which theoretically could shift the metabolic balance towards epoxide formation (Farinola and Piller, 2007).

In the double-blinded study of Vanscheidt *et al.* (2002), 231 patients with chronic venous insufficiency received 90 mg coumarin and 540 mg troxerutin (114 patients) or placebo (117 patients) for 16 weeks. The results of this study were published in separate papers. Schmeck-Lindenau *et al.* (2003) reported on elevated liver enzymes observed in 9 patients. Causal relationship was classified as “unrelated” in 3 cases, as “unlikely” in 2 cases, as “possible” in 3 cases and as “probable” in 1 case. The interpretation regarding coumarin hepatotoxicity is further complicated by elevated liver enzymes also observed in the placebo group (number of

patients with this response not given), probably due to prior hepatopathy (which was not an exclusion criteria) reported in 4 patients in the treatment group and in 2 patients in the placebo group. In addition, the possible hepatoprotective effect of troxerutin has to be considered. Complex logistic regression estimated a basic risk for elevated liver enzymes of 4.9 % in the treatment group and of 2.1 % in the placebo group (risk factors: hepatitis in history and diseases of the liver). Genotyping of CYP2A6 was carried out in all patients, but no evidence was obtained that this is a determinant of coumarin-associated liver dysfunction (Burian *et al.*, 2003).