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1. Introduction (Problem statement)

In 2005 (EMEA, 2005), the HMPC prepared the 'Public statement on the use of herbal medicinal products containing estragole'. There are a large number of plants and their preparations which contain estragole, sometimes in very high amounts. From the European perspective, the most interesting plants are *Foeniculum vulgare* Mill. (both fruit and essential oil) and *Pimpinella anisum* L. (fruit).

HMPC concluded on the basis of the available toxicological data that estragole is a naturally occurring genotoxic carcinogen with a DNA potency similar to safrole. There is a general consensus that the mechanism of action of genotoxicity and carcinogenicity is the dose dependent production of a reactive metabolite, the sulfate conjugate of the 1'-hydroxy estragole, and its subsequent binding to DNA and eventual genotoxic and carcinogenic sequelae. The metabolic activation and DNA binding occur also in human experimental systems. However, as the HMPC concluded, the profiles of metabolism, metabolic activation, and covalent binding are dose dependent and the relative importance diminishes markedly at low levels of exposure (i.e. these events are not linear with respect to dose). In particular, rodent studies show that these events are minimal probably in the dose range of 1-10 mg/kg body weight, which is approximately 100-1000 times the anticipated human exposure to this substance.

For the above reasons HMPC concluded in 2005 that the present exposure to estragole resulting from consumption of herbal medicinal products (short time use in adults at recommended posology) does not pose a significant cancer risk. Nevertheless, HMPC noted the need of further studies to define both the nature and implications of the dose-response curve in rats at low levels of exposure to estragole. In the meantime exposure to estragole of sensitive groups such as young children, pregnant and breast-feeding women should be minimised. Also, toxicological assessment of preparations for topical and external use needs further investigation because data on absorption through the skin are missing.

Since 2005, a number of significant publications on estragole have appeared in the scientific literature. The new data has raised concerns from a toxicological point of view and this has prompted the HMPC to re-assess all available data regarding their relevance for the safe human use of herbal medicinal products containing estragole.

1.1. Estragole in plants and plant preparations

Estragole (1-allyl-4-methoxybenzene, molecular formula: $C_{10}H_{12}O$, molecular mass: 148.20 g/mol, CAS.-No.: 140-67-0) is a volatile phenylpropanoid belonging to a group of alkenylbenzenes such as eugenol, isoeugenol, methyleugenol, safrole, isosafrole, anethole, elemicin, myristicin, apiole. A comprehensive perspective on structural and metabolic variations of alkenylbenzenes was recently published by Rietjens *et al*. (2014).

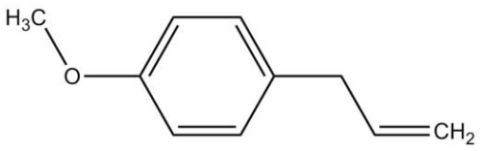


Fig. 1: Structural formula of estragole

Estragole is a major or minor component of a large number of plants or plant parts used for herbal medicinal products, botanicals and flavourings (Iten and Saller, 2004; EFSA, 2009). Table 1 provides some of the most important plants containing estragole. It is of importance to note that many of these plant sources contain a number of other alkenylbenzenes or other components which may affect the

kinetics or dynamics of estragole. These potential matrix effects are being described in appropriate sections when research findings are available.

Table 1: Main occurrence of estragole in plants and/or essential oils (modified from EFSA, 2009, based principally on Council of Europe publications)

Botanical name	**Common name**	**Essential oil in plant (%) / estragole in essential oil (%)**	**Estragole in part of plant used (%)**
Agastache foeniculum (Pursh.) Ktze. (syn. *Lophantus anisatus A. anethiodora, A. anisata*) (Lamiaceae)	Anise hyssop, Giant hyssop, Liquorice mint	? / 74	
Anthriscus cerefolium (L.) Hoffm. ssp *cerefolium* (Apiaceae)	(Garden) chervil	0.9 in fruit/up to 85	maximum 0.8
Artemisia dracunculus L. (Asteraceae)	Tarragon	0.25-1 in herb/60-75	0.7
Foeniculum vulgare Mill. subsp. vulgare var. vulgare (syn. *Foeniculum vulgare* Mill. var. dulce (Mill.) Batt. et Trab.) (Apiaceae)	Sweet fennel, Roman fennel	? / 1.5-5.0	
Foeniculum vulgare Mill. subsp. vulgare var. vulgare (syn. *Foeniculum vulgare* var. vulgare) (Apiaceae)	Bitter fennel, Common fennel	2-6 in fruit/3.5-12.0	0.3
Illicium verum Hook f. (Magnoliaceae)	Star-anise	5 in fruit/5-6 l	maximum 0.25
Melissa officinalis L. (Lamiaceae)	Lemon balm	no info/6.3	
Myrrhis odorata (L.) Scop (Apiaceae)	Sweet chervil	no info/up to 75	
Ocimum basilicum L. (Lamiaceae)	Sweet basil	0.8 in herb/20-89	approximately 0.4
Pimpinella anisum L. (Apiaceae)	Anise, Sweet cumin	1-4 in fruit/1-5	maximum 0.2

In the earlier EMEA public statement (EMEA, 2005) a large number of other plants, mainly essential oils, which contain estragole, were listed.

1.2. Exposure to estragole from herbal medicinal products and food

A major factor of relevance for the risk assessment and actions to take, is to evaluate the background exposure to alkenylbenzenes (and other related and relevant substances) from foodstuffs and food commodities of the consumer. Some official estimates of daily intake of estragole in foodstuffs indicate

that baseline exposures are in the range of 0.5-5 mg estragole per day from the average food intake (Table 2). There probably exist large individual (and possibly regional) differences in estragole intake.

Table 2: Intake of estragole in foodstuffs

Daily exposure	Comments	Reference
4.3 mg	European data (adults)	SCF, 2001
1 mg	approximate estimate, total intake from all sources	CoE, 2005
166 µg, 400-600 µg per day	US population from spice and spice oils - estimate	JECFA, 2009

Presence of estragole in actual preparations has been estimated in two studies. In a study of Bilia *et al.* (2002), fennel teas were prepared by classical infusion or microwave decoction of unbroken and crushed fruits, pre-packaged teabags and instant teas and estragole was analysed by gas chromatography/mass spectrometry (GC–MS). Estragole was present in teas as a minor component, 0.8–4.1% of the total volatiles, but it is not known the extraction percentage from the original preparation. Zeller and Rychlik (2006) determined the extraction efficiency for estragole in an herbal infusion of 12%. The German Chemisches und Veterinäruntersuchungsamt (CVUA, 2007) tested anise and fennel teas. The extraction efficiency was less than 2%. A recent study of van den Berg *et al*. (2014) described the analysis of estragole content in dry fennel preparations and in infusions prepared from them with a special emphasis on extraction efficiency. The range of estragole levels was 0.15-13.3 mg/g in starting dry fennel preparations, whereas the estragole content in infusions was considerably lower ranging between 0.4 and 133.4 µg/25 ml infusion prepared from 1 g dry material (i.e. 0.016-5.34 µg/ml). Extraction efficiency varied between <0.1 to 2.5% in a sample of 37 fennel-based preparations. In addition, the nature of the starting material was important, because infusions prepared from whole fennel fruits contained about 3-fold less estragole compared to infusions prepared from fine-cut fennel material. In general, extraction efficiencies depend on many variable factors and the best estimate for any product or process is probably reached by extraction experiments with the preparation itself. Mihats *et al*. (2016) estimated the daily exposure from consumption of fennel teas (food teas, no information about the content of essential oil) ranging from 0.008-20.78 µg/kg per day (infants), 0.25-5.04 µg/kg per day (children), 0.32-6.42 µg/kg per day (women) and 0.15-2.93 µg/kg per day (men), respectively. Squeezing the bag at the end of the infusion time and using broken fruits increased the content of estragole in the tea preparations.

1.3. Regulatory status

There are currently no limits for estragole in the area of medicinal products.

In 2000 the Committee of Experts on Flavouring substances of the Council of Europe evaluated estragole and recommended a limit of 0.05 mg/kg (detection limit). It was not specified if this limit is related to the intake or to the content of the herbal substance.

Scientific Committee on Food (SCF, 2001) concluded that estragole is both genotoxic and carcinogenic and on this basis recommended reduction in exposure levels and restrictions on use.

The expert panel of the Flavour and Extract Manufacturers Association concluded in 2002 that dietary exposure to estragole from spice consumption does not pose a significant cancer risk to humans because several studies clearly established that profiles of metabolism, metabolic activation and

covalent binding were dose dependent at high levels but diminished markedly at lower levels of exposure (Smith *et al*., 2002).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has been evaluated a group of allyl alkoxybenzenes, including estragole, present in food and essential oils and used as flavouring agents (JECFA, 2009). The Committee concluded that the data reviewed on the six alkoxy-substituted allylbenzenes provide evidence of toxicity and carcinogenicity to rodents administered with high doses for several of these substances. A mechanistic understanding of these effects and their implications for human risk have yet to be fully explored and will have a significant impact on the assessment of health risks from alkoxy-substituted allylbenzenes at the concentrations at which they occur in food. Further research is needed to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents.

2. Discussion

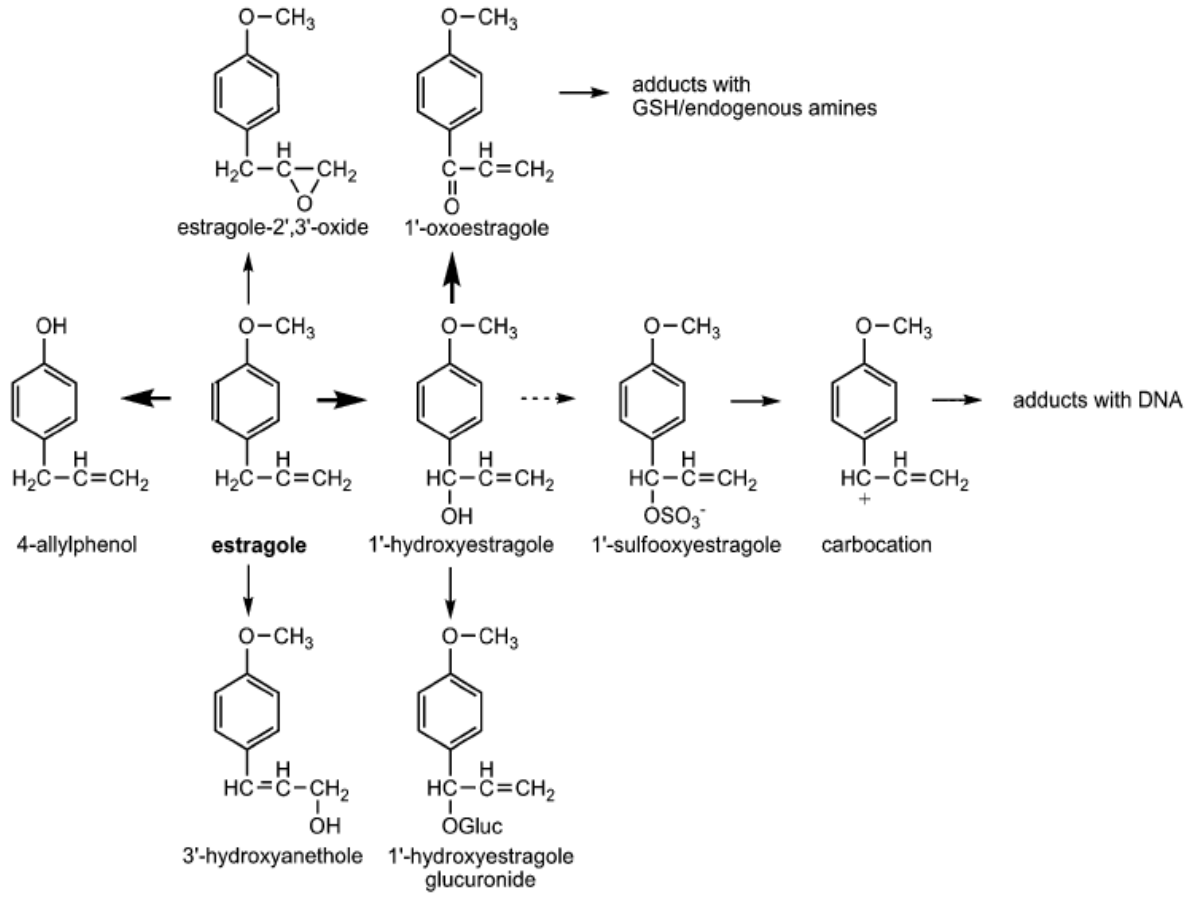
Since 2005, a large number of significant publications on estragole and various alkenylbenzenes have appeared in the scientific literature and prompted HMPC to reassess the toxicology of estragole and of preparations containing these constituents.

2.1. Pharmaco-/toxicokinetics, ADME characteristics

The major metabolic pathways of estragole have been well characterised in rats and mice *in vitro* and *in vivo* and studies have been published on *in vitro* metabolism of estragole in human hepatic preparations (Fig. 2). Three major metabolic pathways have been established:

1. O-demethylation resulting 4-allylphenol and more distal metabolites (and ultimate formation of CO_2). O-demethylation represents a detoxication pathway.
2. 1'-hydroxylation, which is a proximal active metabolite undergoing sulfoconjugation to 1'-sulfooxyestragole capable of binding to DNA and protein. 1'-Hydroxyestragole undergoes also further oxidation to 1'oxoestragole and glucuronidation to 1'-O-glucuronide. The principal enzymes in the bioactivation pathway are CYP1A2 (Jeurissen *et al*., 2007, human and mouse enzymes) and SULT1A1 (Suzuki *et al*., 2012, mouse enzyme).
3. Epoxidation of the allyl side chain leading to estragole-2',3'-epoxide, which is rapidly metabolised by epoxide hydrolase and glutathione transferase to detoxified metabolites (Guenthner *et al*., 2001). This pathway is also regarded as a detoxification route.

There is also the side chain terminal hydroxylation to 4-methoxy-cinnamyl alcohol, but it is not known what is the exact pathway for the formation of this metabolite, i.e whether it is formed via 2,3-epoxidation.



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151 **Fig. 2**: Metabolic pathways of estragole (from Paini *et al*., 2012).

152 Proportions of individual metabolites of different pathways have been proposed to change as a function
153 of dose (Anthony *et al*., 1987). At low doses (in the range of 0.05 to 50 mg/kg bw) O-demethylation
154 predominates, whereas at higher doses (500 and 1000 mg/kg bw) urinary 1'-hydroxyestragole
155 increases relatively. However, urinary concentrations of any single metabolite such as 1'-
156 hydroxyestragole are dependent on both the formation and further biotransformations (and, naturally,
157 other significant pharmacokinetic processes of importance for this particular metabolite) and do not
158 necessarily reflect the concentration of the metabolite available for, e.g. adduct formation. Thus, a
159 more distal marker for activation, e.g. adducts in target molecules, are more reliable evidence for
160 potential dose-dependent change.

161 Concerning humans, it has been reported that after oral administration of estragole to two volunteers
162 (100 µg; single dose) the excretion of 1'-hydroxyestragole in the urine amounted to 0.2 and 0.4% of
163 the administered dose. Other metabolites detected were 4-methylhippuric acid (12%), 4-
164 methoxyphenyllactic acid (4%), 4-methoxycinnamoylglycine (0.8%) and 4-methoxyphenylacetic acid
165 (0.5%) (Sangster *et al*., 1987).

166 Rietjens's group has developed a physiologically-based biokinetic (PBK) model defined by apparent
167 V_{max} and K_m values obtained in *in vitro* microsomal studies for the different phase I conversions of
168 estragole and also for the phase II conversion of 1'-hydroxyestragole (Punt *et al*., 2008, 2009; 2010;
169 Rietjens *et al*., 2010). The performance of the model was analysed based on existing *in vivo* animal
170 and human data. The PBK model was extended into physiologically-based dynamic (PBD) model which
171 would predict the formation of DNA adducts in the liver of male rats on the basis of *in vitro* incubations
172 with rat hepatocytes exposed to estragole (Paini *et al*., 2010). The model was validated using *in vivo*
173 DNA adduct formation in the liver of mice exposed to estragole (Randerath *et al*., 1984). Use of these

models predicts that the formation of the principal adduct in rat liver is linear up to at least 100 mg/kg bw, allowing for the estimation of adduct yields at realistic (human) exposures under certain set of assumptions.

For further validation of the model, Paini *et al*. (2012) quantified the dose-dependent estragole-DNA adduct formation in rat liver and the urinary excretion of 1'-hydroxyestragole glucuronide in male outbred Sprague Dawley rats (n=10, per group), which were administered with estragole once by oral gavage at dose levels of 0 (vehicle control), 5, 30, 75, 150, and 300 mg estragole/kg bw and sacrificed after 48 hours. A dose-dependent increase in DNA adduct formation in the liver was observed. The increase in DNA adduct formation was statistically significant at a dose of 30 mg/kg and interindividual variability was high. In lungs and kidneys DNA adducts were detected at lower levels and mainly at higher doses (>150 mg/kg) than in the liver confirming the occurrence of DNA adducts preferably in the target organ, the liver. The results obtained showed that the PBD model predictions for both urinary excretion of 1'-hydroxyestragole glucuronide and the guanosine adduct formation in the liver were comparable within one order of magnitude to the values actually observed *in vivo*.

2.2. Acute and sub-acute toxicity

Rats given 4 daily doses of 605 mg estragole/kg bw displayed liver injury as observed on gross examination (Taylor *et al*., 1964). In the National Toxicology Program (NTP) study (Bristol, 2011) female mice administered 600 mg estragole/kg body weight died during week 1 because of liver necrosis.

2.3. Sub-chronic toxicity

In the NTP study (Bristol, 2011), male and female F344/N rats and B6C3F1 mice were given estragole (greater than 99% pure) in corn oil by gavage for 3 months. Core and special study (rats only) groups of 10 male and 10 female rats and mice were administered 37.5, 75, 150, 300, or 600 mg estragole/kg bw in corn oil by gavage, 5 days per week. The core study groups were given estragole for 3 months and the special study groups for 30 days.

Rat study

All core study rats survived the 3-month exposure period. Toxicologically the most important findings were observed in serum (increase in ALT, SDH and bile salt) and liver (hepatocellular hypertrophy, bile duct hyperplasia, chronic periportal inflammation). Findings were generally dose-dependent and some responses were observed even at the lowest dose (37.5 mg/kg), where the histological changes included bile duct and oval cell hyperplasia seen in all males and females, and chronic periportal inflammation seen in all males and in 5/10 females, associated with chronic periportal cellular infiltration (histiocyte) in all males but not in females. At two lowest dose levels these changes were judged to be minimal and at higher dose levels their severity was increased. Additionally, two 600 mg/kg male rats had multiple cholangiocarcinomas in the liver and a third had a hepatocellular adenoma.

Other toxicologically significant findings were observed in the erythron (anemia, decrease in total iron binding capacity, reactive thrombocytosis), bone marrow (hyperplasia), kidney (increased weight, tubular histology), the olfactory epithelium (degeneration at 2 highest doses), the *pars distalis* of the pituitary gland (chromofobied cells), submandibular salivary gland (cytoplasmic alterations), gastric glands in the stomach (atrophy), testes and epididymic (degeneration, hypospermia).

In the special study, serum gastrin concentration and stomach pH were significantly increased in rats exposed to 600 mg/kg for 30 days. Gastric gland atrophy was significantly increased in the stomach of

300 and 600 mg/kg rats. Hepatic 7-pentoxyresorufin-O-deethylase activity was significantly increased in all exposed groups except 37.5 mg/kg females, and the increases were generally dose related.

There was no NOAEL/LOAEL value determination in the NTP study. There were minimal histopathological liver changes even at the lowest dose (37.5 mg/kg bw) and these changes were judged to be mostly minimal at the next dose (75 mg/kg bw) and their severity increased at higher dose levels. Thus the lowest dose of 37.5 mg/kg bw could be regarded either a NOAEL or a LOAEL depending on whether the minimal hepatic changes are regarded as toxicologically significant.

Mouse study

In the mouse core study, a 600 mg/kg male died during week 9, and all 600 mg/kg female mice died during week 1; the female deaths were attributed to liver necrosis caused by estragole exposure. In the mouse, liver was the principal target organ based on increased weights, hepatocellular hypertrophy and hepatocellular degeneration, oval cell hyperplasia, and necrosis (all 600 mg/kg female mice). NOAEL level was 37.5 mg/kg bw daily, based on increased liver weights in males and incidence of oval cell hyperplasia in females at 75 mg/kg.

Other significant findings were in the gastric glands of the glandular stomach (degeneration), the forestomach (squamous hyperplasia, mineralisation, and ulcer), and olfactory epithelium (degeneration). These findings were statistically significant at the one of two highest doses.

On the basis of acute and sub-chronic studies, liver is the principal target organ in both rats and mice.

2.4. Chronic toxicity

No animal or human studies have been identified in the literature. Estragole is included into the NTP program.

2.5. Genotoxicity

Prokaryotic tests

Earlier studies have been assessed and summarised by Tice (1999), EMEA (2005), CoE (2005) and EFSA (2009).

Results of mutagenicity testing of estragole in *Salmonella typhimurium* were generally negative, likely due to the complex metabolism required for bioactivation *in vivo*. In the NTP study (Bristol, 2011) estragole was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, or TA1537 when tested in the presence or absence of exogenous metabolic activation enzymes.

Positive results were reported for estragole in strain TA1535 with the addition of the sulphation cofactor 3'-phospho-adenosine-5'-phosphosulphate (PAPS). The putative toxic metabolites of estragole, namely 1'-hydroxyestragole and allyl epoxides of estragole, were generally positive in mutagenicity assays with or without exogenous activation.

Estragole produced mixed results in a DNA repair test, exhibiting dose-related DNA damage in *Bacillus subtilis* in one study and exhibiting negative results in *Bacillus subtilis* and *Escherichia coli* in another.

Eukaryotic in vitro tests

Estragole and its metabolites induced unscheduled DNA synthesis (UDS) in several studies in human and rat cell lines or *ex vivo* in the livers of rats treated orally with estragole.

Martins *et al*. (2012) evaluated the genotoxicity of estragole in V79 cells using the sister chromatid exchange (SCE) assay and the alkaline comet assay and in in two CHO cell lines using the Comet

assay. An increase in SCE without the S9 mix was observed. A positive result was also observed in the alkaline comet assay without S9, indicating DNA strand breakage. In V79 cells a dose-dependent formation of DNA adducts by use of the (32) P-post-labelling assay was observed. Comet assay in two CHO cell lines was positive without biotransformation. The results suggest that estragole, besides being metabolised to genotoxic metabolites, may also be a weak direct-acting genotoxin that forms DNA adducts.

In vivo tests

In the *in vivo* rat study (Nesslany *et al*., 2010), the UDS assay in rat liver was positive, but a bone-marrow micronucleus test was negative.

In the *in vivo* mouse micronucleus test (Bristol, 2011), no increases in the frequencies of micronucleated normochromatic erythrocytes were observed in peripheral blood samples from male and female mice in the 3-month study.

Estragole is clearly genotoxic in transgenic mouse and rat strains (Suzuki *et al*., 2012a, b). For details of these studies, see below.

2.6. Carcinogenicity

No human studies are available.

Mouse studies

In the early studies of the Millers' laboratory (Drinkwater *et al*., 1976; Miller *et al*., 1983; Wiseman *et al*., 1987) estragole or its natural metabolites including 1'-hydroxyestragole or synthetic derivatives administered to adult or newborn mice of different strains (CD-1, B6C3F1, CeH/HeJ, or C57B1/6J) through different routes of administration (diet, oral intubation, ip or sc injection), produced hepatocellular carcinomas. For the carcinogenic potency of estragole in female mice a TD_{50} of 50-100 mg/kg bw resulted from the above studies (CoE, 2005).

Rat studies

A *sc* injection study of derivatives of estragole in male rats did not observe any treatment-related increases in tumours.

In the above mentioned 3-month NTP study (Bristol, 2011), two 600 mg/kg male rats out of 10 animals had multiple cholangiocarcinomas in the liver and a third had an hepatocellular adenoma. The NTP study authors regarded these findings as significant evidence for carcinogenicity of estragole, when all associated evidence including other NTP studies on alkenyl benzenes were taken into consideration.

Further indirect evidence for carcinogenicity of estragole is provided by a recent ToxCast toxicogenomics-based modelling study of Auerbach *et al*. (2010). An ensemble of support vector machine classification models based on male F344 rat liver gene expression following 2, 14 or 90 days of exposure to a collection of hepatocarcinogens (aflatoxin B1, 1-amino-2,4-dibromoanthraquinone, N-nitrosodimethylamine, methyleugenol) and non-hepatocarcinogens (acetaminophen, ascorbic acid, tryptophan) was developed. Independent validation was performed using expression data from the liver of rats exposed at 2 dose levels to a collection of alkenylbenzene flavouring agents. The models differentiated between hepatocarcinogenic (estragole and safrole) and non-hepatocarcinogenic (anethole, eugenol and isoeugenol) alkenylbenzenes previously studied in a carcinogenicity bioassay. The models predict that two alkenylbenzenes not previously assessed in a carcinogenicity bioassay, myristicin and isosafrole, would be weakly hepatocarcinogenic if studied at a dose level of 2 mmol/kg bw per day for 2 years in male F344 rats.

2.7. Reproductive toxicity

No data on reproductive toxicity and teratogenicity are available.

2.8. Mode-of-action (MoA) considerations

The best evidence for a genotoxic mechanism comes from metabolic activation studies: CYP enzymes, especially CYP1A2 (but also others) catalyse the formation of 1'-hydroxyestragole, which, via sulfoconjugation by SULT1A1 and the spontaneous formation of reactive carbocation, binds readily to DNA. Adducts have been characterised both in mice and rats also after *in vivo* exposure to estragole.

On the basis of the above consideration, estragole is a genotoxic hepatocarcinogen and DNA adduct(s) is (are) the first pre-initiation step.

Even if there have been no convincing reports regarding estragole hepatocarcinogenicity in rats, a recent study of Suzuki *et al.* (2012a) suggests a possible involvement of genotoxic mechanisms. They examined hepatocarcinogenicity (GST-P, glutathione S-transferase placental type) and proliferation (PCNA, proliferating cell nuclear antigen) biomarkers, DNA adduct formation and *in vivo* genotoxicity of estragole in the livers of wild and reporter gene-carrying F344 rats. Males were administered 600 mg/kg bw estragole by gavage and sequentially sacrificed at weeks 4, 8 and 16 for GST-P and PCNA immunohistochemistry and measurement of estragole-specific DNA adducts by LC-MS/MS in the livers. GST-P-positive foci increased with time in estragole-treated rats from week 4, PCNA-labelling indices being similarly elevated at both weeks 4 and 8. estragole-specific DNA adducts such as estragole-3'-N(2)-dG, 3'-8-dG and 3'-N(6)-dA were consistently detected, particularly at week 4. In a second study, male F344 gpt delta rats were administered 0, 22, 66, 200 or 600 mg/kg bw estragole for 4 weeks. Gpt (guanine phosphoribosyltransferase) mutant frequency in the liver was increased in a dose-dependent manner, with significance at 200 and 600 mg/kg bw in good correlation with PCNA-labelling indices. Mutation spectra analysis showed A: T to G: C transitions to be predominantly increased in line with the formation of ES-3'-N(6)-dA or 3'-8-dG. These results indicate that estragole could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses.

Suzuki *et al*. (2012b) studied the role of SULT1A1 in the potential carcinogenicity of estragole in mice, by assessing the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency of reporter genes in male and female gpt delta mice treated with estragole at doses of 0 (corn oil), 37.5, 75, 150 or 300 (250 in females) mg/kg bw by gavage for 13 weeks. There is a large sex difference in SULT1A1 activity in the mouse liver, higher in females. In this study the mRNA levels of Sult1a1 in female gpt delta mice were 3-to 6-fold higher than those in the males. The levels of estragole-specific DNA adducts in the females were higher than those in the males at all doses except the highest dose. In addition, mutation frequencies of the gpt gene were significantly increased from doses of 75 mg/kg bw of females, but the increment was observed only at the highest dose in males. There were no changes in the micronucleus test among the groups. The authors suggest that specific DNA modifications by the SULT1A1-mediated carbocation formation and the resultant genotoxicity are key events in the early stage of estragole-induced hepatocarcinogenesis of mice. This finding is in line with earlier studies in which a potent inhibitor of SULT activity pentachlorophenol inhibited estragole-induced hepatocarcinogenicity as well as DNA adduct formation (Fennell *et al*., 1985; Wiseman *et al.,* 1987).

2.9. Estragole alone or in plant-derived complex mixtures

One of the basic questions concerning estragole toxicity is the following: does the matrix (i.e. phytochemical or formulary environment) affect the toxicity of estragole? Recently, Gori *et al.* (2012) analysed the factors and conditions affecting the carcinogenicity of estragole and concluded that the

studies performed thus far give a toxicological profile of estragole as an isolated compound and not the profile risk of the entire complex phytochemical mixture. In their analysis of literature, a multitude of substances in preparations affect the fate and effects of estragole, and probably to the extent that the carcinogenic risk is greatly reduced, if not completely removed.

Rietjens *et al*. (2011) have speculated on the existence of several concepts which may lead to reassessment of risk analysis of complex herbal mixtures. 1) Reactive electrophilic metabolites may have beneficial effects, because they may induce the protective gene expression via the electrophile responsive element (EpRE)-mediated pathways, including Nrf-2 pathway. Especially electrophilic quinone/quinone methide-type metabolites are implicated in this respect (see Boerboom *et al.,* 2006; Lee-Hilz *et al*., 2007). 2) Inhibition of dissolution, uptake, or activation of alkenylbenzenes by flavonoids, an effect conceptualised as a matrix effect.

Rietjens's group has also some *in vitro* evidence for the inhibition of sulfoconjugation of 1'-hydroxyestragole by constituents of the basil extract, the most potent of which was nevadensin (Ki for SULT inhibition 4 nM) (Jeurissen *et al*., 2008; Alhusainy *et al*., 2010). By employing the recently developed PBK model (Paini *et al*., 2010) they predicted that co-administration of estragole at a level inducing hepatic tumours *in vivo* (50 mg/kg bw) with nevadensin results in a considerable inhibition of formation of the ultimate carcinogen 1'-sulfooxyestragole. To validate this finding, estragole and nevadensin were co-administered orally to Sprague-Dawley rats, at a ratio reflecting their presence in basil (Alhusainy *et al.*, 2013). Given the role of the SULT-mediated DNA adduct formation in the hepatocarcinogenicity of estragole, these *in vivo* results suggest that the likelihood of bio-activation and subsequent adverse effects in rodent bioassays may be lower when estragole is dosed with nevadensin compared to dosing of pure estragole. In contrast to the above findings, Müller *et al*. (1994) showed that the genotoxic potential of estragole is not masked by ingredients of basil oil. The genotoxic potentials of basil oil and estragole were compared in the UDS test, using basil oil with an estragole content of 88%, and it was concluded that basil oil induced UDS in the same dose range as estragole (Müller *et al.,* 1994). Obviously basil oil contains a high concentration of estragole and the outcome in herbal products with a lower concentration of estragole could be different regarding attenuation of genotoxicity. Consequently, the matrix effect regarding estragole in various herbal preparations remains somewhat debatable. In a recent review article on combination effects (Rietjens *et al*., 2015), the authors conclude that the matrix-derived combination effect between estragole and nevadensin will be significant at dose levels used in rodent bioassays, but that the effect is predicted to be only limited or even absent at realistic human exposure levels.

A review article by Rietjens *et al*. (2015) states that matrix-derived interactions may occur at all levels of ADME and that the interactions may decrease but also increase the bioavailability and/or toxicity of the compounds of interest.

In conclusion, it seems that there are credible mechanisms or processes, which may affect the manifest toxicity of compounds in the phytochemical matrix. However, clear evidence that these mechanisms are operative in appropriate long-term cancer bioassay conditions, save *in vivo* human situation, is rather hypothetical and further studies are needed. Therefore, at the moment postulated matrix effects do not add substantially to the discussion on a possible practical threshold.

3. Conclusions and Recommendations

3.1. Relevance of experimental toxicities for human risk assessment

Are the tumours observed in animal experiments relevant for human risk assessment?

Hepatocellular tumours, especially adenomas, are often regarded as rodent-specific tumours especially if a rodent-specific mechanism of action (liver enzyme induction) could be elicited. There are some preliminary findings of liver enzyme induction in rats, but on the other hand, there is a lot of evidence for genotoxic mechanism, which on the balance may not be equally rodent-specific and seems more significant or at least better investigated. Hepato-carcinogenicity and proliferation biomarker studies as well as *in vivo* transgene mutation studies provide further evidence for genotoxic mode of action. Consequently, genotoxicity-initiated tumours in animals are probably relevant for human risk assessment.

Is the mode of action for tumour formation relevant for human risk assessment?

For estragole, metabolic activation pathway and DNA adduct formation are amply demonstrated in animals and the same pathway is operative in human *in vitro* systems. There is general consensus that adduct formation is causally related to tumorigenesis, unless there are specific and biologically persuasive reasons to the contrary. Consequently, the mode of action for tumour formation is relevant for humans. Furthermore, several closely related alkenylbenzenes such as methyleugenol and safrole display similar characteristics regarding mode of action and tumour formation.

Are toxicokinetic data (metabolic behaviour, activation etc.) conducive to extrapolation of animal data to humans?

Although toxicokinetics and metabolism of estragole have not been thoroughly studied in humans, there is evidence that under *in vivo* administration of estragole to humans, the liver is exposed to the compound and the first step in metabolic activation, the formation of 1'-hydroxyestragole, takes place. Thus, it is probable that toxicokinetic processes in humans are sufficiently similar to those in rodents in which carcinogenicity has been observed, that extrapolation can be regarded adequately reliable. Further *in vitro* and *in vivo* human studies are needed, but it is anticipated that with the help of a refined PB-toxicokinetic/dynamic model scientifically satisfactory view of estragole toxicokinetics and related dynamics could be developed to help human risk assessment.

3.2. Summary of weight of evidence toxicity risk assessment of estragole

A modified weight-of-evidence (WoE) assessment is formally presented in Table 3 taking into account the findings and argumentations above.

Table 3: Summary of WoE evaluation of genotoxicity and carcinogenicity of estragole

Structure/grouping	Closely related alkenylbenzenes are animal genotoxins and carcinogens (safrole, methyleugenol: IARC class 2B), which provide additional albeit indirect evidence for estragole assessment.
Computational models	structural alert models: no information Machine learning models based on toxicogenomics of a set of hepatocarcinogens and non-carcinogens suggest that estragole is hepatocarcinogenic.
Metabolic activation	Convincing evidence for the activation pathway via hydroxylation and sulphoconjugation in rodent and human *in vitro* systems and in rodents *in vivo*.
DNA binding *in vitro*	Identified adducts in rodent and human hepatocytes.
DNA binding *in vivo*	Identified and measurable adducts in livers of mice and rats.

Genotoxicity *in vitro*	Difficult to demonstrate in conventional prokaryotic assays probably because of special activation pathway; generally low mutagenicity without S-9 mix. Some evidence in eukaryotic systems.
Genotoxicity *in vivo*	Demonstrated in rats and mice by transgene mutation techniques. Micronucleus tests consistently negative, but may not be appropriate for the detection of short-lived reactive metabolites in the liver.
Carcinogenicity	Clear evidence of carcinogenicity in mice. Suggestive, but indirect evidence in rats.
Human information	Metabolic activation pathway present and operative also *in vivo*.
Non-linearity in metabolic activation	Inconclusive evidence of dose-dependent non-linearity of metabolic activation and adduct formation. Biokinetic modelling based on *in vitro* and *in vivo* parameters suggests dose-dependent activation.
Potential matrix effects	Hypothetical. It needs further investigation.
WoE conclusions	Estragole is a genotoxic carcinogen in rodents. The MoA seems to be similar in humans as far as it has been possible to study. Processes resulting in a threshold for genotoxic and carcinogenic actions are possible, but ultimately need further investigations. Exposure to estragole may be assessed as if it is “reasonably anticipated to be a human carcinogen”, i.e. risk assessment paradigm should follow other proven carcinogens (however, ’officially’ no such evaluation and conclusion by IARC or NTP has been made).

3.3. Recommendations

Because of the generally accepted evidence of genotoxic carcinogenicity, exposure to estragole should be kept as low as practically achievable. In the evaluation of herbal medicinal products containing estragole Member States should take steps to ensure that the public are protected from exposure.

Concerning credible threshold mechanisms operative in preventing cancer at low exposures

The existence of mechanisms leading to a dose response that is non-linear or has a practical threshold is increasingly recognized, also for DNA-reactive compounds. These effects may be modulated for example by rapid detoxification before coming into contact with DNA or by effective repair of induced damage. All these factors have been mentioned in the guideline ICH M7 (EMA/CHMP/ICH/83812/2013). With respect to complex herbal preparations, it is of importance to consider that the actual exposure situation possibly creates practical thresholds. There are several factors, which interfere with absorption and bioavailability of other components, inhibit the bio-activation of potential toxicants, scavenge reactive intermediates or protect against toxic mechanisms by rapid detoxication, antioxidation or antimutagenesis (see section 2.9 for further details concerning estragole). The consequence of these protecting mechanisms may be the existence of a practical threshold although at present it is not clear from the available data that a threshold-based risk

assessment can be performed for estragole. Therefore, in the absence of the necessary information, conservatism is justified and calculation of a threshold cannot be performed.

In the case that dose-dependent linearity and/or matrix non-linearity could be shown, the regulatory approach to such compounds can be based on the identification of a No-Observed Adverse Effect Level (NOAEL) and use of uncertainty factors to calculate acceptable limits.

Limit considerations

In the case of estragole, the studies coming from the Millers' laboratory constitute the most convincing case for the genotoxic carcinogenicity of estragole (see section 2.6.). For the carcinogenic potency of estragole in female mice a TD_{50} of 50-100 mg/kg bw resulted from the above studies (CoE, 2005).

Although the more recent NTP 3-month rat study (Bristol, 2011) with a wide dose-response range provides some more information it cannot be used as basis for limit calculations, because it is not a properly designed carcinogenicity study.

In the Carcinogenic Potency Database the TD_{50} of estragole is given with 51.8 mg/kg per day, based on the Miller 1983 study.

According to ICH M7 the acceptable intake would be calculated as such:
TD_{50} (values according to Carcinogenic Potency database) divided by 50,000 and to adjust to a human body weight. Generally, for adults the calculation is done with a body weight of 50 kg[1].

51.8 mg/kg day÷50,000×50 kg body weight=0.052 mg/person per day.

Taking into consideration the argumentation above, the short-term duration of treatment by an herbal medicinal product and an increase in an acceptable daily dose may be determined by calculating the less-than-lifetime exposure according to the ICH M7 scheme. However, the calculation has to be based on the accepted posology of the specific herbal medicinal product taking also into consideration the non-avoidable intake by food.

Taking all the published data on estragole together, a conclusive adequate fit-for-purpose assessment for estragole or rather estragole containing active ingredients in herbal medicinal products seems not to be possible at current time. Open points that require further clarification are e.g. quality of the available studies, questions concerning linearity/non-linearity of effects, possible matrix effects (see above). However, the consideration of the guidance value, which can be calculated according to the guideline M7, should be regarded as a helpful tool for statements e.g. on sensitive patient groups, acceptance of estragole containing excipients or also on the duration of use or acceptable daily doses.

Dietary background

The potential daily intake of estragole via food cannot be ignored especially as consumers/patients are not able to avoid this. Although rigorous and comprehensive estimates of estragole intake via food are not available, values of 0.5-5 mg daily have been presented by various authorities in the EU and the USA during past years (see Table 2). However, the latest publication (CoE, 2005) narrows it to 1 mg/person/day and describes higher values given in literature as overestimation. Data that are more recent are not available. This is especially regrettable since during the last years some regulatory actions were taken in Europe (e.g. Regulation EC 1334/2008) to reduce the intake of estragole. No data are available that allow a comparison with current intake levels. Furthermore, the extraction

[1] For ~18% (average) of the European population the body weight is given with less than 60 kg [EUROPEAN COMMISSION 2006]. These numbers would increase to up to 30%, if only taking into account woman. Therefore, the calculation is linked to a body weight of 50 kg. ICH-Guidelines reflect generally to a human body weight of 50 kg (ICHQ3C; ICH M7).

efficiency of estragole from food items may be very variable and the actual exposure to estragole via food will vary accordingly.

General protective measures

Until further data on estragole carcinogenicity are available, an exposure limit of estragole in herbal medicinal products should be based on the background of human exposure via food. European bodies as CoE (CoE, 2005) or national agencies as BfR (BfR, 2002) have recommended consumers to restrict consumption of estragole-containing herbs and spices beyond their occasional use in kitchen and have demanded industry to reduce the amount of estragole in food as far as possible. This implicates that there is a tolerable estragole exposure from food products, while additional estragole exposure via medicinal products should be kept as low as reasonably possible.

To date, the increase in carcinogenic risk from the life-time intake of estragole containing products is not known. To calculate this tools and data are needed which are not readily available. Involvement of experts from the food area and epidemiologists would be necessary to proceed.

Thus, given the uncertainties mentioned above, at current time an exact limit cannot be defined. Nonetheless, it is concluded, that the intake of estragole from (traditional) herbal medicinal products ((T)HMPs) in the general population should be as low as possible.

Early actions to reduce exposure of humans against estragole are mandatory and should be designed to identify sources that are beyond the range of widely distributed consumption. It is concluded, that the intake of estragole from (T)HMPs in the general population should be as low as possible, which includes a short-time duration of use (maximum 14 days) and a discussion about the single / daily doses necessary according to the risk assessment relevant for the concerned (T)HMP.

Pregnant and breast-feeding women

The usage of estragole containing (T)HMPs in pregnant and breast-feeding women is not recommended if the daily intake of estragole exceeds the guidance value of 0.05 mg/person per day, unless otherwise justified by a risk assessment based on adequate safety data.

If this limit is complied with, section 4.6 of the SmPC of the products concerned should be phrased according to the ‘Guideline on risk assessment of medicinal products on human reproduction and lactation: from data to labelling’ (EMEA/CHMP/203927/2005).

Sensitive groups: Children

The usage of estragole containing (T)HMPs in children is not recommended if the daily intake of estragole exceeds the guidance value of 1.0 µg/kg bw, unless otherwise justified by a risk assessment based on adequate safety data.

Cutaneous use

No data concerning absorption of estragole through the skin exist. In order to ensure that the daily dose of estragole is as low as possible the usage of the product should be restricted to a maximum of 2 weeks and the use should be restricted to intact skin.

The same limits and restrictions (daily dosages, children, pregnant and breast-feeding woman) as mentioned above apply also to cutaneous use unless lower absorption rates for the product (relevant matrix) have been shown. This is justified by the fact that absorption might be greatly influenced by skin conditions and/or excipients.

Acceptance of estragole-containing excipients

The use of estragole containing excipients should be avoided as much as possible in (T)HMPs because it is considered an artificially added source of exposure.
For the usage of estragole containing excipients in (T)HMPs the content of estragole should be reduced by appropriate measures to a content below the guidance value of 0.05 mg/person per day for adults and adolescents and 1.0 µg/kg bw for children, respectively.

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