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Application of the margin of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic
Example: Methyleugenol, CASRN: 93-15-2

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ABSTRACT

The weight of evidence points to weak genotoxic activity of methyleugenol, the putative genotoxic carcinogen being 1′-sulphate ester metabolite. Dose response modelling of the data for methyleugenol gave a BMDL10 for male rat liver adenoma or carcinoma (combined) of 7.9 mg/kg-bw/d following adjustment to daily average doses. The MoEs ranged from 100 to 800 depending on the assumptions used in the exposure estimation.

1. Introduction

Methyleugenol is a compound that occurs naturally in a variety of spices, herbs and essential oils, including clove oil, nutmeg, allspice, pimento, basil, hyacinth, citronella, anise, mace, cinnamon leaves, pixuri seeds, and laurel fruits and leaves. It also has been found in blackberry essence, bananas, black pepper, bilberries and walnuts. Although some methyleugenol-containing essential oils are used in non-food applications, the general population is primarily exposed to methyleugenol through ingestion of foodstuffs and/or products containing methyleugenol-containing essential oils as flavourants.

2. Toxicological data

2.1. Genotoxicity

The weight of evidence points to weak genotoxic activity of methyleugenol. Studies on the chemical analogues safrole and estragole indicate that the 1′-sulphate ester metabolites of molecules in this family are probably key to what genotoxic activity is found (see Section 2.3). Methyleugenol is only weakly or non-mutageneic in bacteria and yeast systems and only with metabolic activation (Dorange et al., 1977; Mortelmans et al., 1986; Schiestl et al., 1989; Sekizawa and Shibamoto, 1982; To et al., 1982, NTP, 1998). It was reported that methyleugenol was unable to induce chromosomal aberrations in Chinese hamster ovary cells (CHO cells) while induced sister chromatid exchanges occurred only in the presence of metabolic activation (S9). Further studies confirmed the non-mutagenicity of methyleugenol in various strains of *Salmonella typhimurium* and in the *Escherichia coli* WP2 uvrA strain with and without metabolic activation (S9) (Sezikawa and Shibamoto, 1982). Methyleugenol was able to induce intra-chromosomal recombination in *Saccharomyces cerevisiae* with and without metabolic activation (Schiestl et al., 1989). Methyleugenol produced sister chromatid exchange in Chinese hamster ovary cells only in the presence of metabolic activation and at near cytotoxic levels. Therefore, the positive findings likely occurred secondary to cytotoxicity in which release of lysosomal nucleases may have resulted in a false positive response (Müller et al., 1994).

Methyleugenol did induce unscheduled DNA synthesis (UDS) in cultured rat hepatocytes (Chan and Caldwell, 1992). Metabolites 1′-hydroxymethyleugenol and 2′,3′-epoxymethyleugenol induced unscheduled DNA synthesis (UDS) in cultured rat hepatocytes. The 1′-hydroxy metabolite is a stronger inducer of UDS than the parent methyleugenol (Chan and Caldwell, 1992). The 1′-hydroxy metabolite and corresponding sulphate esters of allyl alkoxybenzene substances have been shown to form DNA adducts *in vivo* and *in vitro*. 32P-post-labelling experiments with adult female CD-1 mice showed the formation of DNA-adducts with methyleugenol (Phillips et al., 1984). DNA-adducts have not been observed

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at dose levels less than 10 mg/kg-bw above the methods sensitivity limit of 1 adduct in 10(7) to 10(8) nucleotides or ca. 0.3 pmol of adduct/mg DNA.

Evidence for chromosomal damage *in vivo* is weak. Methyleugenol was negative in a micronucleus assay in mice treated by gavage with methyleugenol for 14 weeks at doses up to 1000 mg/kg-bw (NTP, 1998, 2000).

2.2. Carcinogenicity

In rats, the NTP 2-year gavage bioassay (NTP, 2000) gave *clear evidence of carcinogenic activity* of methyleugenol in male and female F344 rats based on increased frequency of hepatocellular carcinoma in rats and increased frequency of hepatocellular adenoma, hepatocholangioma and hepatocholangiocarcinoma) and neuroendocrine tumour, malignant, metastatic, stomach, glandularoendocrine tumours of the glandular stomach in male and female rats. Male rats also showed increased incidence of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibroma or fibrosarcoma. A marginal increase in squamous cell neoplasms of the forestomach may have been related to methyleugenol administration in the female rats.

In mice, the NTP 2-year gavage bioassay gave *clear evidence of carcinogenic activity* of methyleugenol in male and female B6C3F1 mice based on the increased incidence of hepatocellular carcinomas in male and female (the substance also induced hepatocellular adenoma and hepatoblastoma in male and female mice as well as hepatocholangiocarcinoma in the high dosed female mice). Neuroendocrine tumours of the glandular stomach in male mice were also considered related to exposure to methyleugenol.

The role of high dose levels, administered by gavage in causing hepatotoxicity, gastric damage, and malnutrition in both mice and rats has been evoked (Smith et al., 2002). Hepatic tumours occurred in severely damaged livers while the neuroendocrine tumour, malignant, metastatic, stomach, glandularoendocrine tumours were likely to have resulted from endocrine responses to chronic gastric damage. The presence of *Helicobacter hepaticus* in the livers of mice was also thought to have confounded the interpretation of the findings.

By intra-peritoneal injection, high doses of methyleugenol and structurally related allylalkoxybenzene derivatives (e.g. estragole and safrole) and their 1′-hydroxy derivatives are carcinogenic in rodents. This has been observed in several different studies in both newborn and adult mice and rats (Miller et al., 1982, 1983; Wiseman et al., 1987).

2.3. Mode of action

The primary mechanism of carcinogenesis appears to be due to the production of the hepatotoxic sulfate conjugate of the 1′-hydroxy metabolite (Wiseman et al., 1987; Smith et al., 2002). The unstable sulfate hydrolyses to form a reactive electrophilic intermediate (carbonium ion or quinonium cation), which binds to proteins and DNA. Sulfate inhibition studies and unscheduled DNA synthesis (UDS) assays on methyleugenol and related compounds including their 1′-hydroxy metabolites (Boberg et al., 1983; Caldwell et al., 1992; Chan and Caldwell, 1992; Hasheminejad and Caldwell, 1994) provide additional evidence that the sulfate ester of the 1′-hydroxy metabolite is the principal intoxication metabolite in animals.

At low doses, 1′-hydroxylation is not the preferred metabolic route, however, at high doses saturation of extra-hepatic O-demethylation, leads to a relative increase of this route and an increase in the percentage of the 1′-hydroxy metabolite. This change in the balance of metabolic pathways with dose is best demonstrated for methoxyallylbenzene derivatives, notably estragole (e.g. Anthony et al., 1987; Punt et al., 2008, 2009) but is recognised as a general route for the alkoxy-substituted allylbenzenes (JECFA, 2008). At low doses in humans, mice and rats, significant amounts methyleugenol are O-dealkylated. As dose levels increase, 1′-hydroxylation (and epoxidation) increases. The total daily urinary production of the 1′-hydroxy metabolite increases significantly, as much as 6000 times, as the dose of estragole increases from 50 μg/kg bw to 50 mg/kg bw and the metabolism shifts to the CYP-catalysed 1′-hydroxylation pathway (Zangouras et al., 1981; Anthony et al., 1987; Gardner et al., 1997). Data on metabolite identification and quantification for rats given different doses (0.9–600 mg/kg bw) of safrole demonstrate similar effects of dose on metabolic shifting. These studies support the conclusion that the combination of increased dose and metabolic shifting results in significant increases in tissue concentrations and total body burden of the 1′-hydroxy metabolite (Benedetti et al., 1977).

The increase in 1′-hydroxylation has been related to dose-dependent induction of selected CYP-450 isoenzymes (e.g. Sharma et al., 2001; Jeurissen et al. 2006). It appears that at dose levels below 10 mg/kg bw per day, the extent of 1′-hydroxylation is low (Zangouras et al., 1981; Sangster et al., 1987). At higher dose levels induction of CYP1A2 (Jeurissen et al., 2006) and CYP2E1 (Gardner et al., 1997a) and subsequent sulfation (via SULTI and SULTII) are linked sequentially to increased production of the sulfate conjugate of the 1′-hydroxy metabolite. This metabolite is linked to GST depletion and oxidative stress, protein adduct formation, DNA-adduct formation, effects on cell growth, hepatotoxicity and eventually carcinogenicity. In addition to enzyme induction, a principal microsomal protein adduct forms from estragole and methyleugenol at low doses (10 and 30 mg/kg bw per day); at higher doses (100 and 300 mg/kg bw per day), an assortment of protein adducts forms (Gardner et al., 1995, 1996; Wakazono et al., 1998). It should be noted that the formation of protein and DNA adducts in liver is dose dependent (Drinkwater et al., 1976; Swanson et al., 1981; Miller et al., 1982, 1983; Boberg et al., 1983; Phillips et al., 1984; Randerath et al., 1984; Wiseman, 1987; Gardner et al., 1995, 1996; Daimon et al., 1998).

Thus a metabolic threshold for carcinogenesis has been proposed. Although this threshold has not been clearly quantified, the data indicates that 1′-hydroxylation and corresponding adduct formation are minimal in the dose range of 1–10 mg/kg-bw (Smith et al., 2002; JECFA, 2008). Further supporting the hypothesis that a threshold linked to metabolic saturation is occurring is the route and form of elimination of methyleugenol in rats and humans. Routes of elimination at low doses include loss as carbon dioxide via expired air (i.e. arising from O-dealkylation) and excretion of polar metabolites in the urine. At higher doses, the fraction eliminated by expired air decreases while the fraction of non-volatile urinary metabolites increases. (Solheim and Scheline, 1973; Burkey et al., 1999).

Thus four separate factors must be considered in the carcinogenic mode of action of methyleugenol:

(i) There is dose-dependent metabolic shifting to the 1′-hydroxylation pathway.
(ii) There is dose-dependent induction of the enzymes that lead to the 1′-hydroxylation.
(iii) The 1′-hydroxylation pathway leads to the 1′-sulphate which forms protein adducts and which is hepatocytotoxic. The consequent increase in cell-division may contribute to carcinogenesis in the liver.
(iv) The 1′-hydroxylation pathway leads to DNA-adduct formation that is directly linked to the formation of hepatic neoplastic lesions.