**OECD 421 Reproductive Toxicity Screen for SF-002**

We have completed a Reproduction/Developmental Toxicity Screening Test of SF-002 in accordance with OECD Test Guideline (TG) 421 for purposes of dose-range finding and screening for potential effects.[[1]](#footnote-2) We used dietary concentrations of SF-002 up to the European Food Safety Authority (EFSA) maximum feasible dose (MFD) of 5% (w/w) in the diet, or 50,000 ppm, which is equivalent to an estimated mean intake of 3,394 mg/kg-bw/day over 28 days for males, 3,978 mg/kg-bw/day for females during pregnancy, and 7,580 mg/kg-bw/day for females during lactation. The dietary concentrations used resulted in top doses of SF-002 that far exceeded the ECHA 1,000 mg/kg-bw/day limit dose for chemical exposures. Thus, this is a high dose, in particular for a flavour, and represents exposure that is >8,400 times higher than what is likely to occur in humans.[[2]](#footnote-3) Our findings suggest very low potential for reproductive or developmental toxicity of SF-002. Key findings include:

* A decrease in adult male body weight over 28 days was observed only at the highest dietary concentration of 50,000 ppm. This exposure was equivalent to an estimated mean intake of 3,394 mg SF-002/kg-bw/day.
* No effect on maternal body weight or food intake during pregnancy was observed at any dietary concentration up to and including 50,000 ppm, which was equivalent to an estimated mean intake of 3,978 mg SF-002/kg-bw/day.
* A decrease in maternal body weight and food intake during lactation was observed only at 50,000 ppm, which is equivalent to an estimated mean intake of 7,580 mg SF-002/kg-bw/day.
* Small decreases in male and female offspring body weight, concomitant with lower maternal weight gain and food intake during pregnancy, were observed only at 50,000 ppm (estimated maternal mean intake during pregnancy of 3,978 mg SF-002/kg-bw/day).
* No effect on male or female anogenital distance (AGD) was observed on postnatal day 4 and no nipples or areolae were observed in male offspring on postnatal day 13 at any dietary concentration.

Overall, few adverse effects were observed, and only at extremely high dietary concentrations (i.e., 50,000 ppm) of SF-002. These were attributed to diet palatability (decreased body weights in males at an estimated mean exposure of 3,394 mg/kg-bw/day) or were observed concomitant with maternal toxicity in the form of reduced body weight and food intake during lactation following exposure to an estimated mean intake of 7,580 mg/kg-bw/day (decreased body weights in male and female offspring). The lack of effect on male AGD and nipple/areolae retention at any dose level indicates that the test article is not an antiandrogen, even at the highest dietary concentration.

An OECD TG 443 study requires many animals, at high cost and many months to complete. The results of our OECD TG 421 show very low potential for reproductive or developmental toxicity of SF-002. This is further supported by a previously run 90-day (OECD TG 408) that did not indicate reproductive toxicity.

**Background:**

Under the new Smoke Guidance (<https://www.efsa.europa.eu/en/efsajournal/pub/6435>), an OECD TG 443 Extended One-Generation Reproductive Toxicity Study (EOGRTS) is required to fulfil EFSA’s requirements for the reauthorization of smoke flavours. A dose-range finder (DRF) study was needed to select doses for an OECD TG 443 study of Kerry smoke flavour primary product (SFPP) SF-002. A previous 90-day study (OECD TG 408) of SF-002 did not indicate a potential for reproductive toxicity; however, an OECD TG 408 study does not address developmental toxicity. In addition, the OECD TG 408 study of SF-002 did not clearly define a No Observed Adverse Effect Level (NOAEL) for adult rats following 90 days of exposure (i.e., the Study Director concluded that no adverse effects were observed at the highest dose tested). Therefore, we carried out a DRF study, based on the OECD TG 421 Reproduction/Developmental Toxicity Screening Test, for SF-002. Importantly, the OECD TG 421 study also can provide clear indications of potential reproductive and developmental toxicity.

**Objectives:**

The objectives were to provide information for the selection of dietary concentrations of SF-002 for an EOGRTS in rats, and to assess potential reproductive or developmental toxicity of SF-002.

**Study Design:**

The dietary concentrations in this study were selected to be 0, 12,500, 25,000 and 50,000 ppm, based on a prior 90-day repeated dose toxicity study with dietary administration of SF-002 in rats. The highest concentration (5% in the diet, 50,000 ppm) is the EFSA MFD for dietary studies, which is as high as the dose can go before nutritional impacts are made in the diet. Dietary concentrations were not adjusted during the lactation period. Chemical analyses of the diets were performed once during the study and accuracy and homogeneity were confirmed.

The design of the study, based on the OECD TG 421, included 10 male and 10 female Wistar Han rats at each dietary concentration level. The following parameters and end points were evaluated in this study: mortality/moribundity, clinical signs, body weight and food consumption, test material intake, macroscopic examination, organ weights and microscopic examination. The following reproduction/developmental parameters were determined: estrous cycle, mating, pregnancy, and fertility indices, precoital time, number of implantation sites, gestation index and duration, viability and survival indices, parturition, maternal care, sex ratio and early postnatal pup development (mortality, clinical signs, body weights, AGD, areola/nipple retention and macroscopic examination). Detailed materials and methods are provided in the full study report (Annex D-3).

**Results:**

**Parental body weights and food intake**

Males: Over 28 days of exposure, body weights of males in the 50,000 ppm diet group were lower than controls when weighed at days 8, 15, 22, and 28. Males in the 25,000 ppm diet group were nominally lower than controls on the same days, but differences were not statistically significant. Male food intake was not significantly different from controls during the dosing period, although dose-response trends were evident during the periods of days 1-8 and day 8-15, but not later in the study, suggesting diet palatability may have been an issue at the beginning of the study, but then the animals acclimated to the diet later in the study.

Females: Pregnant females gained weight similarly throughout pregnancy (average gestation length of 22 days) and there were no significant differences in weight gain among the diet groups. Likewise, there were no significant differences in food intake among the diet groups during pregnancy. During lactation, females in the 50,000 ppm and 25,000 ppm diet groups gained less weight than controls, but the only statistically significant difference was between the 50,000 ppm diet group and controls on day 13, the last day of lactation on which weights were measured. Food intake during lactation was higher than in pregnancy as expected; however, the 50,000 ppm diet group had significantly lower daily food intake during lactation than controls.

**Offspring body weights**

Pups were weighed on postnatal days 1, 4, 7, and 13. Male and female offspring gained weight in all groups from birth to termination on postnatal day 13.

Males: Male pups in the 50,000 and 25,000 ppm diet groups had significantly lower mean body weight than controls on postnatal days 7 and 13. There were no other statistically significant effects on male offspring body weight.

Females: Female pups in the 50,000 ppm diet group had significantly lower mean body weight than controls on postnatal day 13. There were no other statistically significant effects on female offspring body weight.

**Offspring anogenital distance and male offspring nipple retention**

Anogenital distance was measured on postnatal day 4, and values were normalized with the cube root of body weight. The normalized AGDs were similar in all dietary groups, with no statistically significant differences observed. As normal, no nipples were observed in male offspring on postnatal day 13 in any dietary group.

**Other endpoints assessed (data not shown, unaffected by SF-002)**

Additional adult endpoints evaluated in this study included mortality/moribundity, clinical signs, test material intake, macroscopic examination, organ weights and microscopic examination. No mortality and no test material-related changes were noted for any of these adult endpoints.

Additional reproductive/developmental endpoints included estrous cycle, mating, pregnancy, and fertility indices, precoital time, number of implantation sites, gestation index and duration, viability and survival indices, parturition, maternal care, sex ratio, and early postnatal developmental endpoints (mortality, clinical signs, areola/nipple retention and macroscopic examination). No test material-related effects were observed for any of these additional endpoints.

**Test Material Intake (mean mg/kg-bw/day)**

The dietary concentrations used in this study were combined with the measured food intake by the parental animals to calculate estimated daily intake of SF-002. The highest dietary concentration of SF-002 used in this study was selected to meet the EFSA MFD of 5% (w/w), or 50,000 ppm, in the diet. The estimated mean intakes of SF-002 at the highest dose tested were 3,394 mg/kg-bw/day over 28 days for adult males, 3,978 mg/kg-bw/day for adult females during pregnancy, and 7,580 mg/kg-bw/day for females during lactation, which far exceeded the ECHA limit dose of 1000 mg/kg-bw/day for chemical exposures in an OECD TG 421 or 443 study. As noted in our June 15, 2022, letter, we did not intend to use the results of the DRF to determine NOAELs or Lowest Observed Adverse Effect Level (LOAELs). However, given the relative absence of effects observed in this study at such high doses, NOAELs and/or LOAELs for adult males, adult females, and offspring are provided in Table 1. As shown in the tables, all NOAELs and LOAELs are higher than the ECHA limit dose of 1,000 mg/kg-bw/day. The NOAELs in this study are also higher than the NOAEL of 785 mg SF-002/kg-bw/day determined previously in an OECD TG 408 90-day exposure study in rats submitted in the original dossier submission.

**Table 1.** Adult and developmental NOAELs and LOAELs based on dietary concentrations (ppm) of SF-002 and calculated intake of SF-002 (mg SF-002/kg-bw/day),

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | NOAEL (ppm in diet) | NOAEL (mg/kg-bw/day) | LOAEL (ppm in diet) | LOAEL (mg/kg-bw/day) | Critical Effect |
| Adult male | 25,000 | 1,712 | 50,000 | 3,394 | Body weight |
| Maternal (pregnancy) | 50,000 | 3,978 | N/A | N/A | N/A |
| Maternal (lactation) | 25,000 | 4,054 | 50,000 | 7,580 | Body weight, Food intake |
| Developmental | 12,500 | 1,979 | 25,000 | 4,054 | Male pup body weight |

**Discussion:**

The adverse effects observed in this study included lower body weights in adult males over 28 days of dosing in the 50,000 ppm diet group (i.e., 3,394 mg/kg-bw/day), accompanied by nominally lower food intake than controls. It is possible that this high concentration of the test article in the diet affected palatability; food intake in females during pre-mating was also nominally lower in the 50,000 ppm diet group, but there were no effects on maternal weights or food intake during pregnancy in any dietary group up to and including 50,000 ppm (i.e., 3,978 mg/kg-bw/day). Maternal body weight during lactation was significantly lower in the 50,000 ppm diet group (i.e., 7,580 mg/kg-bw/day). The effects on maternal body weight during lactation, but not during pregnancy, likely reflect the higher food intake (and therefore test article intake during lactation), along with the physiological demands of nursing their litters, indicating that the highest dose reached in the study is the maximum tolerated dose (MTD).

Patterns of postnatal growth of offspring mirrored effects on maternal food intake and weight gain during lactation. Maternal weights during lactation and male pup weights were similarly affected at the two highest dietary concentrations, although not consistently statistically significant. Female pup weights followed a similar if less evident pattern. These findings suggest that the effects on offspring weight were secondary to effects on maternal food intake and weight gain.

The extremely high doses required to elicit effects on any adult or reproductive/developmental endpoint assessed in this study indicate low toxicity of SF-002 and low potential for reproductive or developmental toxicity at dietary intakes that do not cause maternal toxicity [i.e., lower offspring body weights in the 25,000 ppm (i.e., 4,054 mg/kg-bw/day) and higher dietary groups]. The lack of effect on male AGD and nipple/areolae retention at any dose level indicates that the test article is not an antiandrogen, even at the highest dietary concentration.

The most sensitive endpoints in this study were body weight in the adult males and postnatal body weight gain in male offspring. The adult males also showed a tendency for lower food intake during the first two weeks of exposure, suggesting that palatability of the diet may have been initially affected by the smoke flavoring. Food intake in adult males during the third and fourth weeks of exposure was similar in all groups. Female offspring mean body weight in the 50,000 ppm diet group (i.e., 7,580 mg/kg-bw/day) was different from controls only on postnatal day 13. Although male offspring appeared to be more sensitive to the effects of maternal SF-002 exposure on postnatal weight gain, the patterns were similar in male and female offspring.

Overall, this TG 421 Reproduction/Developmental Toxicity Screening Test indicates a low potential for reproductive and developmental toxicity of SF-002. Effects observed were likely related to (1) palatability (decreases in adult male body weight) or (2) maternal toxicity at extremely high doses of SF-002 (decreases female body weights and food consumption during lactation resulting in lower male pup body weights).

1. We acknowledge that our letter dated June 15, 2022, mistakenly stated that we planned to conduct a modified OECD TG 422 study. As noted in the letter, the purpose of the screening study was to inform dose levels for an OECD TG 443 study. The OECD TG 421 study is sufficient and intended for this purpose and is consistent with the notified studies to EFSA. [↑](#footnote-ref-2)
2. Calculated using EFSA’s Dietary Exposure model (DietEx) assuming typical use levels and 95th percentile exposure and adjusted for market share data. [↑](#footnote-ref-3)