**Reproductive Toxicity Screen for SF-006**

We have completed a Reproduction/Developmental Toxicity Screening Test of SF-006 in accordance with OECD Test Guideline (TG) 421 for purposes of dose-range finding and screening for potential effects.[[1]](#footnote-1) We used dietary concentrations of SF-006 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 3,575 mg/kg-bw/day over 28 days for males, 4,191 mg/kg-bw/day for females during pregnancy, and 7,329 mg/kg-bw/day for females during lactation. The dietary concentrations used resulted in top doses of SF-006 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 >12,000 times higher than what is likely to occur in humans.[[2]](#footnote-2) Our findings suggest very low potential for reproductive or developmental toxicity of SF-006. Key findings include:

* No adverse test material related effects on adult male body weight or food intake over 28 days of exposure was observed up to 50,000 ppm. This exposure was equivalent to an estimated mean intake of 3,575 mg SF-006/kg-bw/day. However, it should be noted that in the 25,000 ppm or 1,759 mg/kg-bw/day diet group non-adverse lower body weights were reported.
* No statistically significant effect on maternal body weight and 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 4,191 mg SF-006/kg-bw/day.
* Statistically significant decreased maternal body weight and food intake during lactation was observed in the 50,000 ppm diet group, which was equivalent to an estimated mean intake of 7,329 mg SF-006/kg-bw/day. In addition, statistically significant decreased maternal body weight was observed on days 10-13 of lactation in the 25,000 ppm diet group, which was equivalent to an estimated mean exposure of 3,919 mg SF-006/kg-bw/day.
* Statistically significantly decreased male and female pup body weights were observed in the 50,000 ppm diet group on postnatal day 13, the last day of the study (estimated maternal mean intake during lactation of 7,329 mg/kg-bw/day).
* No effect on male or female anogenital distance (AGD) was observed 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., 25,000 or 50,000 ppm) of SF-006. These were attributed to diet palatability (decreased body weights and food intake in females during lactation at an estimated mean exposure of 7,329 mg/kg-bw/day or decreased body weights at an estimated mean exposure of 3,919 mg/kg-bw/day) or were observed concomitant with maternal toxicity in the form of reduced body weight and food intake in male and female offspring on postnatal day 13 at an estimated mean exposure of 7,329 mg/kg-bw/day). 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-006. 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-006. A previous 90-day study (OECD TG 408) of SF-006 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-006 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-006. 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-006 for an EOGRTS in rats, and to assess potential reproductive or developmental toxicity of SF-006.

**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-006 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 to maintain the dose at the highest concentration tested (i.e., 50,000 ppm). 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 endpoints 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-X).

**Results:**

**Parental body weights and food intake**

Males: No test article related changes in body weight and body weight gain were observed at 12,500 ppm. However, males in the 25,000 and 50,000 ppm diet groups had statistically significantly decreased body weight gain compared to controls over the 28-day treatment period and resulted in lower body weighs at the end of treatment. These body weight changes were accompanied with lower food consumption in the 50,000 ppm diet group. In addition, body weight gain in the 12,500 ppm diet group was transiently lower over days 1-8 and 1-15, but were not considered test article related because the body weights of the males in the 12,500 ppm group were nominally higher than controls at the start of treatment. However, considering the magnitude of change in body weight, this was regarded as non-adverse by the study director.

Females: Pregnant females gained weight similarly throughout pregnancy (average gestation length of 22 days) and there were no statistically 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 diet group gained less weight than controls, but the only statistically significant difference was between the 50,000 ppm diet group and controls on days 1, 4, 7, 10 and 13. Food intake during lactation was higher than in pregnancy as expected; however, the 50,000 ppm diet group had significantly lower daily food intake compared to controls during days 7-10, 10-13 and 1-13. Maternal body weights were lower than controls in the 25,000 ppm diet group on postnatal days 10 and 13; however, food intake was not significantly lower in the 25,000 ppm diet group compared to 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 ppm diet group had statistically significantly lower mean body weights compared to controls on postnatal day 13 only. There were no other statistically significant effects on male offspring body weight.

Females: Female pups in the 50,000 ppm diet group had statistically significantly lower mean body weights compared to controls on postnatal day 13 only. 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 expected, male AGDs were higher than females AGDs in all dietary group and no areolae or nipples were observed in any male offspring on postnatal day 13 in any dietary group.

**Other endpoints assessed (data not shown, unaffected by SF-006)**

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 in 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-006. The highest dietary concentration of SF-006 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-006 at the highest dose tested were 3,575 mg/kg-bw/day over 28 days for adult males, 4,191 mg/kg-bw/day for adult females during pregnancy and 7,329 mg/kg-bw/day for females during lactation, which far exceeded the ECHA limit dose of 1,000 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 1,367 mg/kg-bw/day determined previously in an OECD TG 408 90-day exposure study in rats submitted in the original dossier submission.

**Table 1.** NOAELs and LOAELs for SF-006 based on dietary concentrations (ppm) and estimated mean intake of SF-006 (mg SF-006/kg-bw/day)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | NOAEL (ppm) | NOAEL(mg/kg-bw/day) | LOAEL (ppm) | LOAEL(mg/kg-bw/day) | Critical Effect |
| Adult male | 50,000 | 3,575 | N/A | N/A | N/A |
| Maternal (pregnancy) | 50,000 | 4,191 | N/A | N/A | N/A |
| Maternal (lactation) | 25,000 | 3,919 | 50,000 | 7,329 | Body weight  |
| Developmental (Maternal intake during pregnancy) | 50,000 | 4,191 | N/A | N/A | N/A |
| Developmental (Maternal intake during lactation) | 25,000 | 3,919 | 50,000 | 7,329 | Lower pup weight on PND 13 |

**Discussion:**

The adverse effects observed in this study included lower body weights in lactating females over 28 days of dosing in the 25,000 and 50,000 ppm diet groups (i.e., >3,919 mg/kg-bw/day), accompanied by statistically significantly lower food intake than controls only in the 50,000 ppm diet group. It is possible that this high concentration of the test article in the diet affected palatability. 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). Both male and female pups were similarly affected in the 50,000 ppm diet group (i.e., 7,329 mg/kg-bw/day) and had statistically significantly lower body weights than controls, but only on postnatal day 13. 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-006 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 50,000 ppm group (i.e., 7,329 mg/kg-bw/day)]. 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 decreased body weights in adult females during lactation and postnatal day 13 body weight gain in male and female pups. Overall, this OECD TG 421 Reproduction/Developmental Toxicity Screening Test indicates a low potential for reproductive and developmental toxicity of SF-006. Effects observed were likely related to (1) palatability (decreases in adult female bodyweight and food consumption during lactation) or (2) maternal toxicity at extremely high doses of SF-006 (decreases in pup body weights on postnatal day 13).

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-1)
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-2)