

SCIENTIFIC OPINION

Scientific Opinion on safety of smoke flavour Primary Product – TRADISMOKE™ A MAX¹

EFSA Panel on Food Contact Materials, Enzymes,
Flavourings and Processing Aids (CEF)^{2,3}

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ABSTRACT

This opinion concerns the safety of the smoke flavouring Primary Product TRADISMOKE™ A MAX. The Panel considered the technical and analytical data provided acceptable to characterise the Primary Product and to demonstrate its batch-to-batch variability and stability. Two *in vivo* genotoxicity tests were negative and sufficient to eliminate the concerns over the *in vitro* genotoxicity. The Panel derived a NOAEL of 1000 mg/kg bw/day from a 90-day study in rats. Based on this NOAEL and on the intake data calculated with the use levels of the Primary Product TRADISMOKE™ A MAX provided by the applicant for the 18 food categories, the margins of safety would amount to 30 and 61 for the intake estimates based on the upper use levels, and to 139 and 294 when normal use levels are considered. When assuming the use in traditionally smoked products only, the margins of safety would amount to 83 and 120 for the intake estimates based on the upper use levels, and to 417 and 588 when normal use levels are considered.

The fact that these margins of safety based on a 90-day toxicity study are inadequate, and in addition, that data on reproduction and developmental toxicity and long term studies are absent, it is concluded that the uses and use levels for the Primary Product in a wide range of product categories would require a larger margin of safety. The Panel concludes that the margins of safety are insufficient and that the use of Primary Product TRADISMOKE™ A at the proposed uses and use levels is of safety concern.

KEY WORDS

Smoke Flavouring, Primary Product, TRADISMOKE™ A MAX.

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SUMMARY

The European Food Safety Authority has been asked to provide scientific opinions on the safety of smoke flavouring Primary Products used or intended for use in or on foods. This opinion concerns a smoke flavouring Primary Product, TRADISMOKE™ A MAX.

The Primary Product is obtained solely from beech wood sawdust by controlled pyrolysis. Essential parameters of the manufacturing process have been provided. Water (51 wt. %) functions as solvent of the Primary Product. The total mass (30 wt. %) identified by capillary gas chromatographic and capillary gas chromatographic/mass spectrometric analysis corresponds to 61 % of the solvent-free fraction. The unidentified constituents amount to 19 wt. % of the Primary Product. Except for benzo[*j*]fluoranthene, the concentrations of the polycyclic aromatic hydrocarbons (PAH) known to be carcinogenic and/or genotoxic, listed in the EFSA guidance document, have been provided. The levels of benzo[*a*]anthracene and benzo[*a*]pyrene are below their respective limits of 20 µg/kg and 10 µg/kg as set in Regulation (EC) No 2065/2003. No significant batch-to-batch variability was observed. The stability of the Primary Product was demonstrated by GC analysis of a sample stored at room temperature up to 13 months.

The genotoxic potential of the TRADISMOKE™ A MAX was tested in three *in vitro* studies (a bacterial reverse mutation test, a mouse lymphoma gene mutation assay and a chromosome aberration test) and two *in vivo* genotoxicity assays (a mouse bone marrow micronucleus assay and a Comet assay). These studies were performed according to current OECD guidelines (with the exception of the Comet assay for which no international guideline is yet available) and in compliance with GLP, respectively. The Primary Product was positive in *in vitro* assays for mutagenicity in bacterial and eukaryotic cells, and was clastogenic in CHO cells however; chromosomal aberrations were accompanied by cytotoxicity in CHO cells at high doses. No induction of genotoxicity was observed in the two *in vivo* studies.

Overall, it is concluded that TRADISMOKE™ A MAX is genotoxic *in vitro* in the bacterial reverse mutation test, mouse lymphoma assay and is clastogenic in CHO cells, whereas two *in vivo* genotoxicity tests are negative and sufficient to eliminate the concerns over the *in vitro* genotoxicity.

The TRADISMOKE™ A MAX caused no adverse effects in the 90-day study in rats. The No-Observed-Adverse-Effect level (NOAEL) was 1000 mg/kg bw/day, the highest dose tested.

Use levels of the Primary Product recommended by the applicant are 0.1 to 0.3% (m/m). The maximum use level of the Primary Product in the final food product is recommended to be 0.5 % (m/m).

In order to estimate dietary exposure to the Primary Product TRADISMOKE™ A MAX, the CEF Panel used two different methodologies, developed by the Panel specifically for smoke flavourings. Dietary exposure estimates were calculated by assuming that the Primary Product TRADISMOKE™ A MAX is present at the normal or upper use levels provided by the applicant for the 18 food categories as outlined in Commission Regulation 1565/2000.

Dietary exposures from all sources were 16.3 and 33.8 mg/kg bw/day, when assuming that the Primary Product TRADISMOKE™ A MAX is present at the upper use levels, 3.4 and 7.2 mg/kg bw/day, when normal use levels are considered.

When dietary exposure estimates are based on use in only traditionally smoked foods dietary exposures were 8.3 and 12.0 mg/kg bw/day, when assuming that the Primary Product TRADISMOKE™ A MAX is present at the upper use levels, 1.7 and 2.4 mg/kg bw/day, when normal use levels are considered.

Based on these data it is concluded that when assuming that the Primary Product TRADISMOKE™ A MAX is present at the normal or upper use levels provided by the applicant for the 18 food categories, the margins of safety as compared to the NOAEL of 1000 mg/kg bw/day derived from the 90-day

toxicity study in rats with Product TRADISMOKE™ A MAX amount to 30 and 61 for the intake estimates based on the upper use levels and to 139 and 294 when normal use levels are considered.

When assuming the use of Primary Product TRADISMOKE™ A MAX in traditionally smoked products only the margins of safety would amount to 83 and 120 for the intake estimates based on the upper use levels and to 417 and 588 when normal use levels are considered.

Given i) the fact that these margins of safety are based on a 90-day toxicity study, ii) the absence of data on reproduction and developmental toxicity and iii) the absence of long term studies it is concluded that the uses and use levels of Primary Product TRADISMOKE™ A MAX would require a larger margin of safety. The Panel concludes that the margin of safety is insufficient and that the use of Primary Product TRADISMOKE™ A MAX at the proposed uses and use levels is of safety concern.

To decide whether despite the low margins of safety the use of Primary Product TRADISMOKE™ A MAX might be approved for traditionally smoked products, at use levels specified, to replace smoking, is outside the remit of the Panel.

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BACKGROUND

Smoking is a process traditionally applied to certain perishable foods such as fish and meat. It was originally used for preservation purposes. In addition the process results in sensory changes (colour and flavour) which impart characteristic properties to smoked foods. With the development of other methods the preservative function of smoking decreased in importance over time and the sensory aspects prevailed.

Nowadays, liquid smoke flavourings are added to various foods to replace the smoking process or to impart smoke flavour to foods which are not traditionally smoked.

Smoke flavourings are produced by controlled thermal degradation of wood in a limited supply of oxygen (pyrolysis), subsequent condensation of the vapours and fractionation of the resulting liquid products. The resulting Primary Products (primary smoke condensates and primary tar fractions) may be further processed to produce smoke flavourings applied in or on foods.

The Regulation (EC) No 2065/2003 of the European Parliament and the Council (EC, 2003) established Community procedures for the safety assessment and the authorisation of smoke flavourings intended for use in or on foods. As stated herein the use of a Primary Product in or on foods shall only be authorised if it is sufficiently demonstrated that it does not present risks to human health. A list of Primary Products authorised to the exclusion of all others in the Community for use as such in or on food and/or for the production of derived smoke flavourings shall therefore be established after the European Food Safety Authority (EFSA) has issued an opinion on each Primary Product.

The Guidance on submission of a dossier on a smoke flavouring Primary Product for evaluation by EFSA (EFSA, 2005) lays down the administrative, technical and toxicological data required.

TERMS OF REFERENCE

The EFSA is requested according to Article 8 of Regulation (EC) No. 2065/2003 of the European Parliament and of the Council on smoke flavourings used or intended for use in or on foods to carry out risk assessments and deliver a scientific opinion on the safety of Primary Products.

ASSESSMENT

1. Introduction

The following evaluation applies only to the Primary Product TRADISMOKE™ A MAX manufactured strictly in conformity with the specified process and meeting the chemical specifications described in this opinion.

In accordance with the guidance document (EFSA, 2005), data on the manufacturing process, the composition, intended use levels and toxicological tests have been submitted. The latter include a 90-day oral toxicity study and three *in vitro* genotoxicity tests. Two *in vivo* genotoxicity tests have also been provided.

2. Information on existing authorisations and evaluations

No information on existing authorisations and evaluations of the Primary Product TRADISMOKE™ A MAX has been provided.

3. Technical data

3.1. Manufacturing process

3.1.1. Source materials for the Primary Product

The Primary Product TRADISMOKE™ A MAX is produced from beech (*Fagus grandifolia*) wood sawdust, free of bark particles. Each batch of sawdust is controlled as regards moisture content and every tenth batch is analysed for pesticides by an external certified laboratory. According to a certificate provided by the supplier the wood used is not subjected to chemical treatment.

3.1.2 Method of manufacture of the Primary Product

The production comprises the following steps: (i) pyrolysis under controlled conditions, (ii) condensation by indirect heat exchanger and (iii) filtration and decantation to remove charcoal particles, heavy oil and heavy tar. The pyrolysis is performed in a special electrically heated “Spirajoule” reactor. A flow chart showing the essential steps of the process and indicating the Primary Product has been provided in the application.

Details on the operational parameters have been given. According to the applicant the production process has not been certified but is performed according to ISO 9001 quality assurance rules and controlled by HACCP.

3.2 Identity of the Primary Product

3.2.1 Trade names of the Primary Product

The trade name of the Primary Product is TRADISMOKE™ A MAX.

3.2.2 Physical state of the Primary Product

The Primary Product is described by the applicant as a dark brown liquid with an average density of 1.109 kg/l, a viscosity < 10 cp and an ash content of 1.6 % (600 °C, 6h).

3.3 Chemical composition of the Primary Product

3.3.1 Overall characterisation

The overall characterisation of the Primary Product is as follows:

3.3.1.1 Solvent-free fraction

Water functions as the solvent of the Primary Product TRADISMOKE™ A MAX. A water content of 51 wt. % was determined by Karl Fischer titration. Accordingly, the solvent-free fraction of the Primary Product amounts to 49 wt. % (Figure 1).

3.3.1.2 Volatile fraction

The Primary Product was analysed by capillary gas chromatography (GC). Mass spectrometry (MS) was used for identification and flame ionisation detection (FID) for quantification. On average the volatile fraction determined by GC amounted to 31 wt. %, of which up to 1 wt. % remained unidentified. Accordingly, 97 % of the volatile fraction were identified which is in compliance with Commission Regulation (EC) 627/2006 (EC, 2006).

3.3.1.3 Unidentified constituents

The unidentified constituents amount to 19 wt. % of the Primary Product. The total identified mass (30 wt. %) corresponds to 61 % of the solvent-free fraction (Figure 2). This is in compliance with Commission Regulation (EC) 627/2006 (EC, 2006).

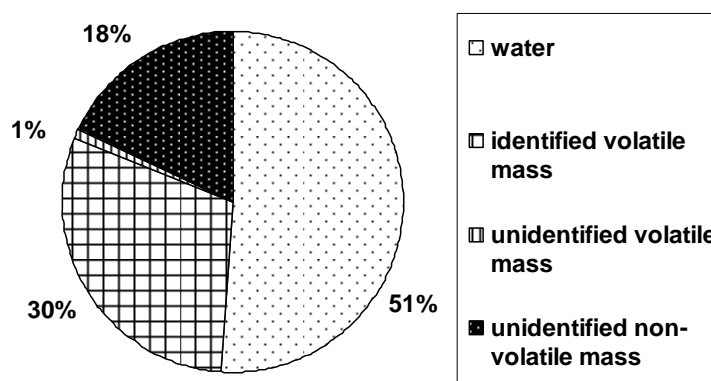


Figure 1. Overall composition of TRADISMOKE™ A MAX (wt. % of Primary Product)

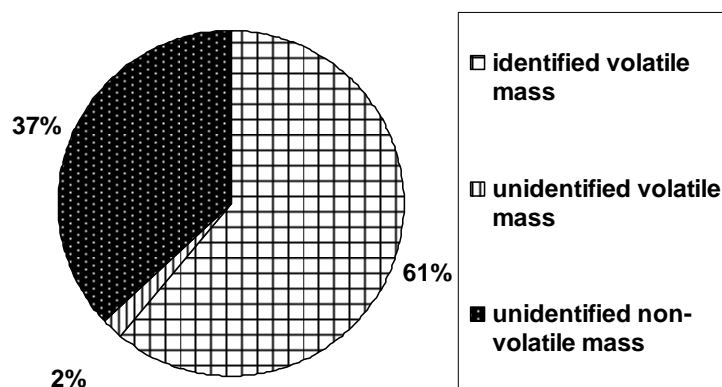


Figure 2. Composition (%) of the solvent-free fraction of TRADISMOKE™ A MAX

3.3.2 Chemical description of the Primary Product

The Primary Product has been characterised according to the parameters listed in Table 1. The applicant provided detailed descriptions of the analytical methods.

Table 1. Description of major chemical parameters of the Primary Product¹

Acidity, as acetic acid (%)	14.9 ± 1.4
Carbonyls, as heptanal (%)	19.9 ± 2.6
Phenols, as 2,6-dimethoxyphenol (%)	36.2 ± 5.2
pH	2.1

¹No information on the number of analysed batches was provided

For one batch the following contents of heavy metals were provided: arsenic < 0.2 mg/kg, mercury < 0.03 mg/kg, cadmium < 0.05 mg/kg and lead < 0.2 mg/kg.

3.3.3 Identification and quantification of Primary Product constituents

2.3.3.1 Principal constituents

The Primary Product was analysed by GC and GC/MS. In total, 49 constituents amounting to 30 wt. % were identified. The proportions of the 25 principal constituents are listed in Table 2.

Table 2. Principal constituents of the Primary Product (lot COA/0066)^a

Compound	Concentration (g/l)
Acetic acid	112
1-Hydroxy-2-propanone	69
1-Hydroxy-2-butanone	16
Levoglucozan	13
Formic acid	14
Methanol	12

Allyl dimethoxyphenol (isomer I)	12
5-Methyl-2-furancarboxaldehyde	6.5
Methyl acetate	5.9
Hydroxyacetaldehyde	5.3
Furfural	5.4
Syringol	5.0
Methoxy eugenol	4.2
3-Methyl-1,2-cyclopentanedione	3.5
Allyl dimethoxyphenol (isomer II)	3.4
2(5H)Furanone	3.0
Methyl Syringol	2.9
Pentanal	2.9
Propanoic acid	2.6
1,4:3,6-Dianhydro- α -D-glucopyranose	2.5
Desaspidinol (2',6'-Dihydroxy-4'-methoxybutanophenone)	2.5
Pyrocatechol	2.3
1-Acetyloxy-2-propanone	2.2
1,2-Cyclopentanedione	2.2
3-Hydroxy-3-methylbutanoic acid	2.0

^a Data obtained by duplicate analyses of three samples of the batch

3.3.3.2 Content of Polycyclic Aromatic Hydrocarbons (PAHs)

Except for benzo[*j*]fluoranthene, the concentrations of the polycyclic aromatic hydrocarbons (PAHs) known to be carcinogenic and/or genotoxic, listed in Annex 2 of the guidance document (EFSA, 2005) have been provided. The analyses were performed by an external accredited laboratory; the method used was equivalent to the method developed by the Joint Research Centre of the European Commission (Simon *et al.*, 2006a and b), except for the analyte benzo[*j*]fluoranthene. The concentrations of the 14 PAHs determined in the Primary Product are listed in Table 3.

The levels of benzo[*a*]anthracene and benzo[*a*]pyrene are below their respective limits of 20 $\mu\text{g}/\text{kg}$ and 10 $\mu\text{g}/\text{kg}$ in Regulation (EC) No 2065/2003 (EC, 2003).

Table 3. Concentrations of PAHs in the Primary Product (lot COA/0062)

PAH	Concentration (µg/kg)
Chrysene	3.7
Benzo[<i>a</i>]anthracene	2.7
5-Methylchrysene	< 1
Cyclopenta[<i>cd</i>]pyrene	1.7
Benzo[<i>b</i>]fluoranthene	1.2
Benzo[<i>k</i>]fluoranthene	< 1
Benzo[<i>a</i>]pyrene	1.9
Indeno[<i>1,2,3-cd</i>]pyrene	< 1
Dibenzo[<i>a,h</i>]anthracene	< 1
Benzo[<i>ghi</i>]perylene	2
Dibenzo[<i>a,e</i>]pyrene	< 1
Dibenzo[<i>a,h</i>]pyrene	< 1
Dibenzo[<i>a,i</i>]pyrene	< 1
Dibenzo[<i>a,l</i>]pyrene	< 1

3.3.4 Batch-to-batch variability

Six batches of the Primary Product were analysed by GC/FID and GC/MS to demonstrate batch-to-batch variability. Area counts relative to an internal standard were determined for 66 components. The average relative standard deviation was 15.5 %. The individual values ranged from 2.5 % for major products (acetic acid; 25 area %) to 113 % for minor components (2,4- and 2,5-dimethylphenol; 0.05 area %).

3.3.5 Stability

The stability of the Primary Product was checked by subjecting a batch stored in a standard plastic container for about one year at room temperature to GC analysis for four times (after 1, 4, 7 and 13 months). 66 components were analysed for absolute and relative standard deviation. The average relative standard deviation determined for 66 components was 10 %. No consistent increases or decreases were observed.

3.3.6 Specifications

Specifications of the Primary Product as given by the applicant on the technical data sheet are shown in Table 4.

Table 4. Specifications of the Primary Product TRADISMOKE™ A MAX

Density	1.1 kg/l
pH	1.5 - 2.5
Total acidity (in acetic acid equivalent)	13 - 16 wt. %
Carbonyls	17 - 22 wt. %
Phenols	30 - 45 mg/ml
Benzo[<i>a</i>]pyrene	<10 ppb
Benzo[<i>a</i>]anthracene	<20 ppb
Arsenic	<3 mg/kg

Mercury	<1 mg/kg
Cadmium	<1 mg/kg
Lead	<10 mg/kg

According to the applicant, the shelf-life of the Primary Product is 12 month under cool and dry conditions.

4. Proposed uses

Normal and upper use levels as described by the applicant for the Primary Product in each of the 18 food categories as outlined in Commission Regulation (EC) No 1565/2000 (EC, 2000) are reported in Table 5.

Table 5. Normal and upper use levels of Primary Product in food categories as outlined in Commission Regulation (EC) No 1565/2000 applicant

Food categories		Use level (g/kg)	
		Normal	Upper
1	Dairy products, excluding products of category 2	0	0
2	Fats and oils and fat emulsions (type water-in-oil)	1	1
3	Edible ices, including sherbet and sorbet	0	0
4.1	Processed fruits	0	0
4.2	Processed vegetables (including mushrooms & fungi, roots & tubers, pulses & legumes) and nuts and seeds	0	0
5	Confectionery	0	0
6	Cereals and cereal products, including flours & starches from roots & tubers, pulses & legumes, excluding bakery	0	0
7	Bakery wares	0	0
8	Meat and meat products, including poultry and game	1	5
9	Fish and fish products, including molluscs, crustaceans and echinoderms	1	5
10	Egg and egg products	0	0
11	Sweeteners, including honey	0	0
12	Salts, spices, soups, sauces, salads, protein products etc.	1	5
13	Foodstuffs intended for particular nutritional uses	0	0
14.1	Non-alcoholic ("soft") beverages, excl. dairy products	0.1	0.1
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts	0.1	0.1
15	Ready-to-eat savouries	1	5
16	Composite foods (eg. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 - 15	1	5

5. Exposure Assessment

In order to estimate dietary exposure to the Primary Product, the CEF Panel used two different methodologies, developed by the Panel specifically for smoke flavourings (EFSA, 2009).

The Smoke Theoretical Added Maximum Daily Intake (SMK-TAMDI) is an adaptation of the Theoretical Added Maximum Daily Intake (TAMDI) method used by the Scientific Committee on Food (SCF) to assess exposure to single flavouring substances (Scientific Committee for Food, 1995). As for the TAMDI, the SMK-TAMDI also assumes that the hypothetical consumer will daily consume a fixed amount of flavoured solid foods and liquids. However, in the SMK-TAMDI approach a single group "Beverages" is used for liquids whereas solid foods are divided in "traditionally smoked solid foods" and "other solid foods not traditionally smoked".

The European Prospective Investigation into Cancer and Nutrition (EPIC) study is one of the few cases in which the consumption levels of “smoked meat” were assessed for different European countries (Linseisen *et al.*, 2006). The CEF Panel used consumption data from the EPIC study to estimate the potential cumulative dietary exposure to smoke flavourings. The smoke flavouring EPIC model (SMK-EPIC) is based on a number of assumptions, in particular it assumes that a hypothetical high consumer of smoked meat is also an average consumer of the other traditionally smoked foods and an occasional consumer of smoked foods or beverages from each of the other categories.

Dietary exposure estimates were calculated by assuming that the Primary Product is present at the normal or upper use levels provided by the applicant for the 18 food categories as outlined in Commission Regulation (EC). When the normal use levels are used, the SMK-TAMDI can be considered as an adaptation of the modified TAMDI (mTAMDI), the method used by the AFC Panel (EFSA, 2004) to screen and prioritise flavouring substances.

Details of the methodologies are described in the dietary exposure document (EFSA, 2009).

Dietary exposure estimates calculated by means of the above mentioned methods are reported in Tables 6. Dietary exposures from all sources were 16.3 and 33.8 mg/kg bw/day, when assuming that the Primary Product is present at the upper use levels, 3.4 and 7.2 mg/kg bw/day, when normal use levels are considered.

The impact on exposure of using the Primary Product only in traditionally smoked food products was also assessed. Out of the above mentioned 18 food categories, “Dairy products, excluding products of category 2”, “Meat and meat products, including poultry and game” and “Fish and fish products, including molluscs, crustaceans and echinoderms” were considered as “Traditionally smoked solid foods”. In this case the SMK-EPIC model results in the highest exposure estimates: 2.4 and 12.0 mg/kg bw/day when using normal and upper use levels, respectively.

Table 6. Summary of the dietary exposure estimates to the Primary Product

Methodologies		Dietary exposure (mg/kg bw/day)	
		Normal use levels	Upper use levels
SMK-TAMDI	Traditionally smoked food	1.7	8.3
	Other foods not traditionally smoked	5.0	25.0
	Beverages (alcoholic or non-alcoholic)	0.5	0.5
	Total dietary exposure	7.2	33.8
SMK-EPIC	Traditionally smoked food	2.4	12.0
	Other foods not traditionally smoked	0.9	4.1
	Beverages (alcoholic or non-alcoholic)	0.1	0.1
	Total dietary exposure	3.4	16.3
Applicant	Dietary exposure	- ^a	- ^a

^a Not provided

6. Toxicological data

6.1. Identity of the test material

The same batch (number 200403001, produced on 16/03/2004) was used for toxicological tests and the GC-MS study performed by one laboratory. The same batch was used for analysis of

benzo[*a*]anthracene and benzo[*a*]pyrene by a different laboratory. Two other batches (number 0A/090105 produced on 09/01/2005 and number 0A/040405 produced on 04/04/2005) were used for PAH analysis.

Batch COA066 was used for the Comet assay. Data on the major constituents and PAHs were provided to demonstrate the representativeness of this batch of the Primary Product.

6.2. Subchronic toxicity

A 90-day oral toxicity study of TRADISMOKE™ A MAX was performed in CRL(WI)BR male and female rats (LAB, 2005a). The study was performed according to OECD guideline 408 (1998) and in compliance to GLP.

The test item was administered to 10 animals/sex/dose group by oral gavage on 7 days per week at doses of 100, 350 and 1000 mg/kg bw/day using solutions of 10, 35 and 100 mg/ml prepared in 1 % aqueous methylcellulose containing 4 % polysorbate 20, respectively. Samples from all dosing solutions were taken for analysis on first and last treatment days.

Control 1 group was treated with 1 % methylcellulose containing 4 % polysorbate 20, control 2 group received 1 % aqueous methylcellulose only. The evaluation was performed by comparison with values of control 1 group.

One male and three female animals from the high dose group died on day 64, 16, 32 and 53, respectively. The histopathological examination revealed acute tracheitis in all cases. The authors of the study report attributed these results to the administration procedure.

The average body weight of male rats in the 1000 mg/kg bw/day group was slightly below the value for control group 1 (between -1% and -7%) during the whole observation period with statistical significance on days 14, 21, 56 and 63, but this effect was small. The body weight was comparable in all experimental groups of female rats. The mean daily food intake of male rats in the 1000 mg/kg bw/day group was less than that in the control group 1 on weeks 1 and 2 (-10% and -8%, statistically significant, respectively). However, no statistically significant changes in food intake were observed in the following weeks. Food consumption of females was comparable in all groups. No eye alterations were found in ophthalmoscopic observations.

Some statistically significant alterations in haematological parameters were observed. The partial thromboplastin time (PPT) was increased in males at all doses (24%, 4%, 7% in low, mid and high dose, respectively, compared to control), however, statistical significance was reached only at low dose. PPT was also statistically significantly increased in females (47%) at the low dose but no changes were observed at higher doses. The platelet count (PLT) was increased in females (7%, 10%, 24%), however, statistical significance was reached only at high dose and no significant changes were observed in males. The prothrombin time (PT) was statistically significantly increased (43%) at the low dose, but PT was decreased at higher doses (-1%, -4%, not statistically significant) and no significant changes were observed in males. The authors of the study report stated that all these changes were in the physiological range of this species and strain.

Some statistically significant changes of parameters from clinical chemistry were reported. Concentrations of creatinine were decreased in males (-16%, -7%, -7%) but statistical significance was reached only at low dose and no significant changes were observed in females. The concentration of cholesterol was statistically significantly increased in males at low dose (18%) but decreased at higher doses (-4%, -10%, not statistically significant, respectively) and no significant changes were observed in females. The activity of alkaline phosphatase (ALP) was increased in males in low and mid dose (22%, 10%) but reached statistical significance only at low dose and ALP was decreased (-10%, not statistically significant) at the high dose and no significant changes were observed in females. The activity of alanine aminotransferase (ALT) was increased in females in the low and high dose groups (14%, -1%, 59%) but this increase reached statistical significance only at the low dose (there was a high standard deviation in the high dose group (61.71 ± 47.36 U/L)) and no significant

changes were observed in males. The level of phosphate was elevated (dose-related) in males (2%, 9%, 31%, statistically significant at mid and high dose) and females (4%, 8%, 21%, statistically significant at high dose). The authors considered these alterations within normal ranges of this rat strain.

Urinalysis revealed minor changes of pH in males (3%, 0%, -9%, statistically significant at high dose) but these changes were not dose-related and no significant effects were observed in females. No macroscopic alterations related to the test item were found at gross pathology. Organ weights (relative and absolute) were not altered in a dose-related manner and no test item-related findings were observed in histopathological examinations. Thus, under the conditions of this study the test material caused no adverse effects. Hence the authors of the study report concluded that, the NOAEL is 1000 mg/kg bw/day, the highest dose tested.

6.3. Genotoxicity

The genotoxic potential of the TRADISMOKE™ A MAX was tested in three *in vitro* and two *in vivo* genotoxicity assays as required by the Guidance on submission of a dossier on a Smoke Flavouring Primary Product for evaluation by EFSA (EFSA, 2005). All genotoxicity studies were conducted according to current OECD guidelines (except for the Comet assay, for which no international guidelines are yet available) and in compliance to GLP.

The TRADISMOKE™ A MAX induced gene mutations in a bacterial assay performed in accordance with OECD guideline 471 (LAB, 2005b) in which dose-related increases of revertant colony numbers were observed in *S. typhimurium* TA 100 in the presence of metabolic activation (up to 3.2-fold compared to solvent control).

The Primary Product was tested in a L5178Y TK +/- mouse lymphoma assay (performed in accordance with OECD guideline 476) (SPL, 2005). Four-hour exposures were used both with and without metabolic activation. The dose range of the test material, plated for expression of mutant colonies, was selected on the results of a preliminary toxicity test and was 30, 60, 90, 120, 150 and 180 µg/ml in the absence of metabolic activation and 60, 120, 180, 240, 300 and 360 µg/ml in the presence of metabolic activation. The Primary Product induced statistically significant and dose-related increases in the mutant frequency in L5178Y mouse lymphoma cells both in the absence (up to 2.6-fold at moderate cytotoxicity of 0.44 Relative Total Growth (RTG)) and presence of metabolic activation (up to 4.4-fold at 0.34 RTG).

The increase in mutant frequency was predominantly due to the formation of small colonies suggesting clastogenic activity.

In an *in vitro* chromosomal aberration test performed in accordance with OECD guideline 473 (LAB, 2005c), where the test item was applied at concentrations of 5, 25 and 50 µg/ml there was a dose-related increase in the percentage of CHO cells showing structural chromosome aberrations both in the absence (up to 5.3-fold) and presence of metabolic activation (up to 2.7-fold). However, the medium and high concentrations were accompanied by cytotoxicity (about 60% and 17% relative survival compared to solvent control).

In vivo, the Primary Smoke Condensate was tested in a mouse micronucleus assay (LAB, 2004) performed in accordance with OECD guideline 474. The Primary Product was administered once orally to male and female mice (15 animals per sex per group) at 500, 1000 and 2000 mg/kg bw. The bone marrow was sampled at 24, 48 and 72 hours. The Primary Product induced a weak but statistically significant and dose-related increase in the percentage of micronucleated polychromatic erythrocytes in bone marrow of female mice (up to 1.7-fold compared to control at 48 h). The PCE/NCE ratio was not affected. The increases in micronucleated polychromatic erythrocytes in females were statistically significant at 1000 ($p < 0.05$) and 2000 mg/kg bw ($p < 0.01$) at 48 hours. The laboratory stated that these effects were within the historical control ranges but they were within the criteria for a positive result defined by the testing laboratory. However, this test material was not

considered by the testing facility to be clastogenic under the conditions of this *in vivo* assay, although a strict application of the criteria specified for a positive result in the report would suggest this study should be considered positive.

A statistical report was provided to EFSA the applicant in 2007 comparing the mean of the statistically significant positive groups with the range of the negative historical control groups. As the mean of the positive groups was within the historical control range this analysis concluded that the observation falls within the biological acceptable ranges. The Assessment Methodology Unit of EFSA was asked to review this report on behalf of the Panel. It was concluded that the analysis provided by the applicant had some flaws in its statistical approach. Therefore, the slides were re-evaluated in parallel by two experts of an independent laboratory (Microptic Cytogenetic Services, Swansea, UK). The result of the re-evaluation confirmed that the study result was negative (LAB, 2008).

The Primary Smoke Condensate was tested in an *in vivo* Comet assay by Institut Pasteur de Lille, Lille Cedex, France (IPL, 2009), according to GLP. The Primary Smoke Condensate was applied to male Sprague Dawley rats (4 per group). Methylcellulose at 1% in distilled water supplemented with 4% of polysorbate was used as vehicle. The Primary Product was investigated on liver and duodenum cells under alkaline conditions (SCGE) in the male rats, treated orally twice with 500, 1000 and 2000 mg/kg bw/day with one sampling time 3 to 6 hours after the last treatment. The treatments (gavage) were separated by 24 hours. The two highest doses were assessed for genotoxicity. Three slides were prepared for each animal, with four animals per group and 50 cells per slide were scored, *i.e.* 150 cells per animal (600 cells per treatment). The Olive Tail Moment (OTM) was used to evaluate DNA damage. The nuclei's OTM, individual values of Tail Length and % DNA in Tail were given. In addition, each slide was also examined for presence of ghost cells (also known as clouds or hedgehogs). Such ghost cells which were considered an indicator of toxicity and/or apoptosis were recorded but were excluded from image analysis data collection. Although at present, there is no OECD guideline available for this assay, the study design was in agreement with recent international recommendations. Neither clinical signs nor mortality were noted up to 24 hours after the first or second treatment. The cellular viability was assessed using the Trypan blue exclusion method. All calculated relative viabilities were superior to 70 %. The values obtained for the negative control group were inside the limits of historical control data. The positive control 1,2-dimethyl hydrazine induced a median OTM of 8.40 (statistically significant) in hepatocytes and a median OTM of 10.24 in duodenum cells after a single oral application of 10 mg/kg bw/day. In hepatocytes, the median OTMs for 600 cells were 0,05, 0,08 and 0,10 at 0, 1000 and 2000 mg/kg bw/day dose levels, respectively. In duodenum cells, the median OTMs were 1,68, 1,26 and 1,58 at 0, 1000 and 2000 mg/kg bw/day dose levels, respectively. The results observed after application of Primary Smoke Condensate were not statistically significantly different from control and were inside the limits of historical control data. Thus, under the experimental conditions applied, the primary Smoke Condensate did not induce statistically significant increases in DNA strand breaks at any dose tested in liver or duodenum cells of rats. The Primary Smoke Condensate was not genotoxic in this assay.

6.4. Other studies

No other studies on TRADISMOKE™ A MAX were provided by the applicant.

DISCUSSION

The Panel considered the technical and analytical data sufficient to characterise the Primary Product and to demonstrate its batch-to-batch variability and stability.

The Primary Product was positive in *in vitro* assays for mutagenicity in bacterial and eukaryotic cells and was clastogenic in CHO cells, however, chromosomal aberrations were accompanied by cytotoxicity in CHO cells at high doses. The Primary Smoke Condensate was tested in an *in vivo* micronucleus assay and an *in vivo* Comet assay. The results of these *in vivo* assays were negative.

Overall, it is concluded that TRADISMOKE™ A MAX is genotoxic *in vitro* in the bacterial reverse mutation test, mouse lymphoma assay and is clastogenic in CHO cells, whereas two *in vivo* genotoxicity tests are negative and sufficient to eliminate the concerns over the *in vitro* genotoxicity.

The TRADISMOKE™ A MAX was investigated in a 90-day study in rats. Four animals died in this study, however, the Panel agreed with the authors of the study report that this was not related to the test item. Some statistically significant effects were observed on body weight, some haematological and clinical chemistry parameters and in urinalyses. However, the Panel agreed with the authors of the study report that these effects generally occurred sporadically, were in most cases not dose-related and were within the physiological ranges of this species and strain. The NOAEL was 1000 mg/kg bw/day, the highest dose tested.

In order to estimate dietary exposure to the Primary Product TRADISMOKE™ A MAX, the CEF Panel used two different methodologies, developed by the Panel specifically for smoke flavourings. Dietary exposure estimates were calculated by assuming that the Primary Product TRADISMOKE™ A MAX is present at the normal or upper use levels provided by the applicant for the 18 food categories as outlined in Commission Regulation 1565/2000. Dietary exposures from all sources were 16.3 and 33.8 mg/kg bw/day, when assuming that the Primary Product TRADISMOKE™ A MAX is present at the upper use levels, 3.4 and 7.2 mg/kg bw/day, when normal use levels are considered.

When dietary exposure estimates are based on use in only traditionally smoked foods dietary exposures were 8.3 and 12.0 mg/kg bw/day, when assuming that the Primary Product TRADISMOKE™ A MAX is present at the upper use levels, 1.7 and 2.4 mg/kg bw/day, when normal use levels are considered.

Based on these data it is concluded that when assuming that the Primary Product TRADISMOKE™ A MAX is present at the normal or upper use levels provided by the applicant for the 18 food categories, the margins of safety as compared to the NOAEL of 1000 mg/kg bw/day derived from the 90-day toxicity study in rats with Product TRADISMOKE™ A MAX amounts to 30 and 61 for the intake estimates based on the upper use levels and to 139 and 294 when normal use levels are considered (Table 7).

When assuming the use of Primary Product TRADISMOKE™ A MAX in traditionally smoked products only the margins of safety would amount to 83 and 120 for the intake estimates based on the upper use levels and to 417 and 588 when normal use levels are considered (Table 7).

Table 7. Margins of safety

	Use level	Dietary exposure* (mg/kg bw/day)	NOAEL (mg/kg bw/day)	Margin of safety*
Total dietary exposure	Normal	3.4 / 7.2	1000	294 / 139
	Upper	16.3 / 33.8	1000	61 / 30
Traditionally smoked food	Normal	2.4 / 1.7	1000	417 / 588
	Upper	12.0 / 8.3	1000	83 / 120

* The first figure refers to dietary exposure estimated on the basis of the Smoke-EPIC model; the second one refers to dietary exposure estimated on the basis of the Smoke-TAMDI model.

The Panel did not anticipate that smoke flavourings would be used in food specifically designed for infants (0-12 months) and children (12-36 months). Therefore, the safety of use of Primary Product TRADISMOKE™ A MAX in such products was not assessed.

Given i) the fact that these margins of safety are based on a 90-day toxicity study, ii) the absence of data on reproduction and developmental toxicity and iii) the absence of long term studies it is concluded that the uses and use levels of Primary Product TRADISMOKE™ A MAX would require

a larger margin of safety. The Panel concludes that the margin of safety is insufficient and that the use of Primary Product TRADISMOKE™ A MAX at the proposed uses and use levels is of safety concern.

To decide whether despite the low margins of safety the use of Primary Product TRADISMOKE™ A MAX might be approved for traditionally smoked products, at use levels specified, to replace smoking, is outside the remit of the Panel.

CONCLUSIONS

The Panel considered the technical and analytical data sufficient to characterise the Primary Product and to demonstrate its batch-to-batch variability and stability.

The Primary Product TRADISMOKE™ A MAX is genotoxic *in vitro* in the bacterial reverse mutation test, mouse lymphoma assay and is clastogenic in CHO cells, whereas two *in vivo* genotoxicity tests are negative and sufficient to eliminate the concerns over the *in vitro* genotoxicity.

TRADISMOKE™ A MAX caused no adverse effects in a 90-day rat study. The NOAEL was 1000 mg/kg bw/day, the highest dose tested.

Based on these data it is concluded that when assuming that the Primary Product TRADISMOKE™ A MAX is present at the normal or upper use levels provided by the applicant for the 18 food categories, the margins of safety as compared to the NOAEL of 1000 mg/kg bw/day derived from the 90-day toxicity study in rats with Product TRADISMOKE™ A MAX amounts to 30 and 61 for the intake estimates based on the upper use levels and to 139 and 294 when normal use levels are considered.

When assuming the use of Primary Product TRADISMOKE™ A MAX in traditionally smoked products only the margins of safety would amount to 83 and 120 for the intake estimates based on the upper use levels and to 417 and 588 when normal use levels are considered.

Given i) the fact that these margins of safety are based on a 90-day toxicity study, ii) the absence of data on reproduction and developmental toxicity and iii) the absence of long term studies it is concluded that the uses and use levels of Primary Product TRADISMOKE™ A MAX would require a larger margin of safety. The Panel concludes that the margin of safety is insufficient and that the use of Primary Product TRADISMOKE™ A MAX at the proposed uses and use levels is of safety concern.

To decide whether despite the low margins of safety the use of Primary Product TRADISMOKE™ A MAX might be approved for traditionally smoked products, at use levels specified, to replace smoking, is outside the remit of the Panel.

DOCUMENTATION PROVIDED TO EFSA

1. Dossier submitted by Sofral, May 2005.
2. Response from Sofral to request for supplementary information.

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ABBREVIATIONS

AFC	Scientific Panel on Additives, Flavourings, Processing aids and Materials in Contact with Food
ALP	Alkaline Phosphatase
ALT	Alanine AminoTransferase
bw	body weight
CEF	Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary
EC	European Commission
EFSA	European Food Safety Authority
EPIC	European Prospective Investigation into Cancer and Nutrition
GC/MS	Gas Chromatography/Mass Spectrometry
GI	Gastro Intestinal
GLP	Good Laboratory Practice
GS/FID	Gas Chromatography/Flame Ionisation Detection
HACCP	Hazard Analysis and Critical Control Points
mTAMDI	modified TAMDI
NOAEL	No-Observed-Adverse-Effect Level
OECD	Organisation for Economic Cooperation and Development
OTM	Olive Tail Moment
PAHs	Polycyclic Aromatic Hydrocarbons
PLT	Platelet count
PPT	Partial Tromboplastin Time
PT	Prothrombin Time
RTG	Relative Total Growth
SCF	Scientific Committee on Food
SCGE	Single-cell gel electrophoresis
SMK-EPIC	Smoke flavouring EPIC model
SMK-TAMDI	Smoke Theoretical Added Maximum Daily Intake
TAMDI	Theoretical Added Maximum Daily Intake