PHILIP MORRIS
INTERNATIONAL

D-LIMONENE

SUBSTANCE INFORMATION DOCUMENT

Prepared by:
Toxicological Assessment
Philip Morris International

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D-LIMONENE

MODULE 1

SUBSTANCE INFORMATION SHEET

D-limonene

CAS number	5989-27-5
Natural Origin	Occurs naturally in apples, citrus fruits, fennel, tomatoes and other.
Chemical Formula	C10H16
Synonyms	d-p-mentha-1,8-diene; limonene; 4-isopropenyl-1-methylcyclohexene
E number	N/A
FEMA GRAS number	2633

General Information

Council of Europe (CoE)

Number	Comment
491	N/A

US Food & Drug Administration (FDA)

Number	Comment
21 CFR 182.60	Approved by the U.S. FDA as GRAS

Joint FAO/WHO Expert Committee on Food Additives (JECFA)

Number	Comment
1326	ADI: "not specified". An ADI "not specified" was established for d-limonene by the Committee at its forty-first meeting (Annex 1, reference 107), which was maintained at the sixty-third meeting.

European Food Safety Authority (EFSA)

Number	Comment
01.045	Aliphatic and aromatic hydrocarbons.

Flavors & Extracts Manufacturers Association (FEMA)

Number	Comment
2633	Generally recongnized as Safe as a flavor ingredient - GRAS 3.

Uses and Exposure

d-limonene is reported to be used by the food industry in beverages, meat products, baked goods, candies, puddings and frozen dairies.

Estimated Intake from Food and Drink

The daily intake was estimated by JECFA at 655ug/day in Europe and at 212ug/day in the USA [1].

Summary of the Toxicological Investigations on the Use of the Substance in Tobacco Products

Smoke chemistry

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3 ppm on the composition of the cigarette smoke was investigated in an ingredient mixture study.

Neutral Red Uptake Assay (NRU)

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3 ppm on the cytotoxicity, as measured by the Neutral Red Uptake assay, was investigated in an ingredient mixture study.

Ames Assay

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3 ppm on the mutagenic response, as measured by the Salmonella reverse mutation assay, was investigated in an ingredient mixture study.

Mouse Lymphoma Assay (MLA)

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3 ppm on the on the mutagenic response, as measured by the Mouse Lymphoma Assay, was investigated in an ingredient mixture study.

In vivo Micronucleus

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3ppm on the clastogenic / aneugenic response was

		investigated using the in vivo Micronucleus Assay in an ingredient mixture study.

Inhalation studies

Internal Studies	Levels Tested ppm	Comment
Roemer for Philip Morris	1, 3	The effect of the addition of d-limonene as mix at concentrations up to 3 ppm on the toxicity of cigarette smoke, as suggested in a 90-day inhalation study, was investigated in an ingredient mixture study.

References

1. JECFA Safety evaluation of certain food additives. Sixty-third meeting. WHO Food Additives Series: 54. Geneva, 2006.

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MODULE 2.1

TOXICOLOGICAL DATA AVAILABLE

Toxicological data on unburnt ingredient

Source	Date	Document
N/A	N/A	N/A

Volatile Organic Compounds Analysis

Source	Date	Document
N/A	N/A	N/A

Purge and Trap / Pyrolysis

Source	Date	Document
N/A	N/A	N/A

Transfer Studies

Source	Date	Document
N/A	N/A	N/A

Smoke chemistry

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

In vitro toxicology: Salmonella reverse mutation assay (Ames)

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

In vitro toxicology: Neutral Red Uptake (NRU)

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

In vitro toxicology: Mouse Lymphoma Assay (MLA)

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

In vivo Micronucleus Assay (MN)

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

Inhalation Studies

Source	Date	Document
Roemer for Philip Morris	2014	ROEMER FOR PHILIP MORRIS Toxicological assessment of kretek cigarettes: Part 1. Background, assessment approach, and summary of findings.

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MODULE 2.2

TOXICOLOGICAL DATA SUMMARY

2.2.1 Volatile Organic Compounds Analysis

N/A

2.2.2 Pyrolysis

N/A

2.2.3 Transfer Studies

N/A

2.2.4 Smoke Chemistry

Rationale

Quantitative chemical analysis can provide information on the concentration on selected smoke constituents. Due to the high complexity of cigarette smoke, where a recent listing identified more than 5300 constituents (Rodgman and Perfetti, 2009)[1], the information will always be limited. As a basis for smoke constituent analysis, PMI uses the list presented in the Canadian Tobacco reporting regulations (Health Canada Tobacco Control Programme, 2004)[2].

Method

Mainstream Smoke Generation and Sample Collection
The cigarettes are smoked on smoking machines according to the currently valid ISO standard 3308 (ISO, 2000)[3]. Because the various target compounds are present in both the particulate phase and the gas phase of smoke, the use of various kinds of collection devices is required which are specific to the analyte being measured.

Result

ROEMER FOR PHILIP MORRIS
Mainstream smoke from experimental kretek cigarettes with three ingredient mixes at low and high inclusion rates was compared to a control kretek cigarette of identical construction, but without the addition of ingredients.The mainstream smoke composition was characterized by a comprehensive set of 55 analytes. There were a few sporadic significant differences between control and test cigarettes, but nearly all of them were smaller than ±20% and there was generally no relationship between smoke constituent yield and added ingredient levels. As the TPM deliveries of the test and control cigarettes were similar, the small differences between the cigarettes remain the same as on a per cigarette basis when expressed in percentage terms. Addition of ingredients to test kretek cigarettes including d-limonene at levels up to 3 ppm did not discernibly alter the smoke chemistry profile of the selected major toxic constituents of smoke. The lack of differences in smoke composition of the experimental cigarettes with and without ingredients is mirrored by a lack of differences in the in vitro toxicity.

[1] Rodgman A, Perfetti TA. The chemical components of tobacco and tobacco smoke. 2009. CRC Press.
[2] Health Canada Tobacco Control Programme. Tobacco reporting regulations. 2004.
[3] ISO, International Organisation for Standardisation: International Standard ISO 3308. 2000. Routine analytical cigarette-smoking machine. Definitions and standard conditions, 4th Edition.

2.2.5 *In Vitro* Toxicology: *Salmonella* reverse mutation assay (Ames)

Rationale

The Bacterial reverse mutation assay (Ames assay) as described by Maron and Ames (Maron and Ames, 1983)[4] is a widely used short-term bacterial in vitro genotoxicity assay specifically designed to detect point mutations induced by chemicals and complex environmental and biological mixtures. The assay is recommended by OECD and ICH as part of the standard testing battery for genotoxicity.
The ICH guideline states with regard to genotoxicity assays: "These tests should enable a hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA…is generally considered to be essential for heritable effects and in the multi-step process of malignancy. Compounds which are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens, i.e., may induce cancer and/or heritable effects" (ICH, 1997)[5].
The response to TPM is different in different bacterial strains and with and without metabolic activation. The strains that are most sensitive towards TPM are TA98 and TA100 with metabolic activation. Strains TA102 and TA1535 (with and without metabolic activation) show little or no response to TPM. Strains TA98 and TA1537 without metabolic activation are just above the borderline followed by increasing response by TA100 without S9 and TA 1537 with S9 (Roemer et al., 2002)[6].

Method

In the plate incorporation version of the assay, the test substance and the bacteria with or without the metabolic activation system S9 are mixed in a liquid top agar overlay containing a small amount of histidine to allow for a few cell divisions, and plated immediately on minimal glucose agar plates. These plates are incubated for 2 to 3 days and only those bacteria which revert from histidine auxotrophy to prototrophy can grow up to colonies. The number of induced revertant colonies is the measure of the mutagenic activity of the test substance.
In each assay, concurrent positive controls (strain-specific, S9-specific, and test substance-specific) and negative (solvent) controls with and without metabolic activation are evaluated and used to confirm the assay performance.

Result

ROEMER FOR PHILIP MORRIS
Mainstream smoke from experimental kretek cigarettes with three ingredient mixes at low and high inclusion rates was compared to a control kretek cigarette of identical construction, but without the addition of ingredients. The smoke was assessed in vitro for mutagenicity in the Salmonella typhimurium reverse mutation assay with and without metabolic activation. On a per cigarette and on per mg TPM basis, mutagenicity results for TPM of the test kretek cigarettes containing an ingredient mix were very similar to those of the control kretek cigarette, for both low and high inclusions. There were no cases of significant differences from the control kretek cigarette. This

[4] Maron D, Ames B. Revised methods for the Salmonella mutagenicity test. Mutation Research. 113, 173-215, 1983.
[5] ICH, Genotoxicity: A standard battery for genotoxicity testing for pharmaceuticals. 1997. ICH. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.
[6] Roemer E, Tewes FJ, Meisgen TJ, Veltel DJ, Carmines EL. Evaluation of the potential effects of ingredients added to cigarettes. Part 3: in vitro genotoxicity and cytotoxicity. Food Chem.Toxicol. 40, 105-111, 2002.

study demonstrates within the sensitivity and the specificity of the test systems that the addition of d-limonene at concentrations up to 3 ppm and as part of a mixture, added to Kretek cigarettes did not increase the in vitro mutagenic response of kretek cigarette smoke as measured by the Salmonella reverse mutation assay.

2.2.6 *In Vitro* Toxicology: Neutral Red Uptake (NRU)

Rationale

The Neutral Red Uptake assay developed by Borenfreund and coworkers (Babich H. and Borenfreund E, 1992; Borenfreund and Puerner, 1985)[7] has been widely used by the chemical and pharmaceutical industries as an in vitro screening method to determine the cytotoxicity of compounds (NTP, 2001)[8]. The assay is a well-established, reproducible standardized short-term assay which responds to cytotoxic compounds in a dynamic range of concentrations. (Borenfreund E. et al., 1998)[9].

Method

Cytotoxicity is determined in the neutral red uptake assay in basic accordance with the INVITTOX protocol No. 3a (INVITTOX, 1990)[10]. Mouse embryo BALB/c 3T3 cells clone A31 (ATCC CCL 163) are seeded and cultivated in 96-well microtiter plates in DMEM with 10% fetal bovine serum (FBS). After 24 hours of cultivation, the cells are exposed for 24 h to the smoke fractions dissolved in DMEM with 5% FBS. At the end of exposure, the culture medium containing the smoke fractions is replaced with medium containing the vital dye Neutral Red. After a 3-h incubation period, the Neutral Red, which is taken up only by viable cells, is determined photometrically after extraction from the cells.

Result

ROEMER FOR PHILIP MORRIS
Mainstream smoke from experimental kretek cigarettes with three ingredient mixes at low and high inclusion rates was compared to a control kretek cigarette of identical construction, but without the addition of ingredients. The smoke was assessed in vitro for its cytotoxicity in the Neutral Red Uptake assay (particle phase and gas/vapor phase separately) in mouse embryo BALB/c 3T3 cells.In the cytotoxicity study, there were neither on per cigarette basis nor on per TPM basis any significant differences between the mean 1/EC50 values for ingredient mixes compared with the appropriate control cigarette data, for both the TPM and the gas/vapor phase (GVP). This study demonstrated within the sensitivity and the specificity of the test systems, that the addition of d-limonene to kretek cigarettes at concentrations up to 3 ppm as part of a mixture, had no significant effect on the in vitro cytotoxicity of varying concentrations of kretek cigarette smoke fractions.

[7] Babich H, Borenfreund E. Cytotoxic and morphological effects of phenylpropanolamine, caffeine, nicotine, and some of their metabolites studied in vitro. Toxicol Vitro. 6, 493-502, 1992.
Borenfreund E, Puerner JA. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol Lett. 24, 119-124, 1985.
[8] NTP, Report of the international workshop on in vitro methods for assessing acute systemic toxicity. 2001. Research Triangle Park, NC, National Toxicology Program.
[9] Borenfreund E, Babich H, Martin-Alguacil N. Comparisons of two in vitro cytotoxicity assays - the neutral red (NR) and tetrazolium MTT tests. Toxicol Vitro. 2, 1-6, 1998.
[10] INVITTOX, INVITTOX protocol 3a, the FRAME modified Neutral Red uptake cytotoxicity test. 1990. Nottingham, UK.

2.2.7 *In Vitro* Toxicology: Mouse Lymphoma Assay (MLA)

Rationale

The mouse lymphoma thymidine kinase assay (MLA) has been optimized to quantitatively determine the in vitro mutagenicity of cigarette mainstream smoke particulate phase[11]. The MLA quantifies genetic alterations that affect the expression of the thymidine kinase (tk) gene in L5178Y/tk+/−-3.7.2C mouse lymphoma cells. As the MLA is able to detect a wide range of genetic alterations, i.e., point mutations, larger scale chromosomal changes, recombination, mitotic non-disjunction, and others, it has been regarded as the most sensitive in vitro mammalian cell gene mutation assay and thus especially useful for test substances with unknown or multiple genotoxic mechanisms. Most of these types of alterations are found in human tumor cells and are presumably relevant for carcinogenesis.

Method

The mutagenicity determination was carried out in the mouse lymphoma TK assay with and without the metabolic activation system S9. For each cigarette type, two batches of total particulate matter were prepared and each batch was assayed at three total particulate matter concentrations.

Result

ROEMER FOR PHILIP MORRIS
Mainstream smoke from experimental kretek cigarettes with three ingredient mixes at low and high inclusion rates was compared to a control kretek cigarette of identical construction, but without the addition of ingredients. The smoke was assessed in vitro for mutagenicity/genotoxicity in the mammalian cell mouse lymphoma thymidine kinase assay in L5178Y cells with and without metabolic activation.Results on a per cigarette basis showed no significant differences between the mutagenicity of TPM from the test cigarettes containing either ingriedient mix when compared to that of the control kretek cigarette. On a per mg TPM basis, mutagenicity results for TPM of the test kretek cigarettes containing an ingrident mix were very similar to those of the control kretek cigarette, for both low and high inclusions as well as with and without metabolic activation. There were no cases of significant differences from the control kretek cigarette. This study demonstrates within the sensitivity and the specificity of the test systems that the addition of d-limonene at concentrations up to 3 ppm and as part of a mixture, added to Kretek cigarettes did not increase the in vitro mutagenic/genotoxic response of kretek cigarette smoke as measured by the Mouse Lymphoma assay.

2.2.8 *In Vivo* Micronucleus Assay (MN)

Rationale

The purpose of the micronucleus assay is to identify substances that produce cytogenetic damage and may result in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. The assay detects damage to the chromosomes or the mitotic apparatus of

[11] Schramke, H., T. J. Meisgen, et al. (2006). "The mouse lymphoma thymidine kinase assay for the assessment and comparison of the mutagenic activity of cigarette mainstream smoke particulate phase." Toxicology 227(3): 193-210.

erythroblasts by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells. The in vivo assay is recommended by the International Conferences on Harmonization of the Toxicological Requirements for Registration of Pharmaceuticals for Human Use (ICH, 1995; ICH, 1997)[12] and by the Food and Drug Administration as guidance on testing requirements for food and color additives (MacGregor et al., 2000)[13].

Method

Clastogenicity/aneugenicity are investigated in rats exposed to cigarette smoke according to the procedures outlined in OECD guideline 474 (OECD, 2009)[14]. For the analysis of peripheral blood, at least 20,000 CD-71 reticulocytes (RET) are scored for micronuclei (identified as MnRET) using a flow cytometer (Dertinger et al., 2000)[15]. For the analysis of bone marrow, the frequency of micronucleated polychromatic erythrocytes (MnPCE) in 2,000 bone marrow PCEs is determined using a light microscope or alternatively a flow cytometric approach. Cyclophosphamide serves as a positive control for the assay.

Result

ROEMER FOR PHILIP MORRIS
An in vivo rat micronucleus assay was performed to evaluate the impact of three different ingredient mixes on in vivo clastogenicity/aneugenicity end points of mainstream smoke of test kretek cigarettes in comparison to MS from a kretek control cigarette. Exposure of male rats to MS from test kretek cigarettes containing any of the ingredient mixes did not increase the proportions of micronucleated cells in peripheral blood and in bone marrow over the proportion of micronucleated cells in the control kretek cigarette group. Furthermore, no relevant differences in the proportion of micronucleated reticulocytes and polychromatic erythrocytes were seen between the smoke-exposed groups and the sham-exposed groups. Therefore, MS from these cigarettes, some of them including d-limonene up to 3 ppm, is not considered to be clastogenic/aneugenic in the in vivo micronucleus assay.

2.2.9 Inhalation Studies

Rationale

The 90-day rat inhalation study has demonstrated to be a useful assay for evaluation of inhaled materials including cigarette smoke (Coggins et al., 1989a; Coggins et al., 1989b; Coggins et al., 1993; Gaworski et al., 1997; Gaworski et al., 1998; Vanscheeuwijck et al., 2002)[16].

[12] ICH, Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, adopted by CPMP, Sep. 1995[S2A]. 1995. Genf, ICH. ICH Harmonised Tripartite Guideline.
ICH, Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals. 1997. ICH. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.
[13] MacGregor JT, Casciano D, Muller L. Strategies and testing methods for identifying mutagenic risks. Mutat Res. 455, 3-20, 2000.
[14] OECD, Guideline for testing of chemicals. Test No. 413: Subchronic inhalation toxicity: 90-day study. 2009. Paris, France, Organization for Economic Co-operation and Development.
[15] Dertinger SD, Torous DK, Hall NE, Tometsko CR, Gasiewicz TA. Malaria-infected erythrocytes serve as biological standards to ensure reliable and consistent scoring of micronucleated erythrocytes by flow cytometry. Mutat Res. 464, 195-200, 2000.
[16] Coggins CR, Ayres PH, Mosberg AT, Sagartz JW, Burger GT, Hayes AW. Comparative inhalation study in rats using a second prototype of a cigarette that heats rather than burns tobacco. Inhalation Toxicology 1, 197-226, 1989a.

Among the extended list of endpoints investigated, several have been shown to be reproducibly responsive to cigarette mainstream smoke, with most endpoints related to the irritant properties of smoke. Sensitive endpoints suitable for quantitation of systemic toxicity and/or irritancy include clinical observations, body weight development, weight of selected organs, respiratory physiology parameters, body temperature, and histopathology of the respiratory tract (nose, larynx, trachea, and lung). The respiratory tract has been identified as the primary site of (histopathological) response to smoke inhalation; thus, emphasis should be placed on histopathological changes in the respiratory tract in the inhalation studies (Dalbey et al., 1980; Wehner et al., 1981; Coggins et al., 1992)[17].
For cigarette smoke, an inhalation period lasting 90 days has been shown to be sufficient to obtain pronounced and reproducible smoke-related effects. On the other hand, extending the inhalation period beyond 90 days has been shown to not produce any additional biologically significant histopathological findings or any progression of these respiratory tract lesions (Coggins et al., 1981)[18].
While the assay method typically used by Philip Morris International has been designed to maximize the sensitivity for cigarette smoke, the basic protocol and study designs have been described in OECD guidelines (OECD, 2009)[19] and are amenable to assessment of other materials intended for exposure via the inhalation route.

Method

Cigarettes are smoked on smoking machines, with the test article (e.g., mainstream smoke) directed to the breathing zone of the animals. Typically, rodents (generally male and female rats) are nose-only exposed to either fresh air (sham) or test article. Note: More specific details routinely used for cigarette smoke generations are described by Vanscheeuwijck and coworkers (Vanscheeuwijck et al., 2002)[20].
Exposures may be carried out using various timeframes depending upon the study design. Most of the studies have been performed according to OECD guidelines with 6 hours/day, 7 days/week for 90 days. However, because it could be shown that in general for cigarette smoke inhalation Haber's

Coggins CR, Ayres PH, Mosberg AT, Sagartz JW, Burger GT, Hayes AW. Ninety-day inhalation study in rats, comparing smoke from cigarettes that heat tobacco with those that burn tobacco. Fundam Appl Toxicol . 13, 460-483, 1989b.
Coggins CR, Ayres PH, Mosberg AT, Sagartz JW, Hayes AW. Comparative inhalation study in rats using cigarettes containing tobacco expanded with chlorofluorocarbon-11 (CFC-11) or hydrochlorofluorocarbon-123 (HCFC-123). Inhalation Toxicology 5, 97-115, 1993.
Gaworski CL, Dozier MM, Gerhart JM, Rajendran N, Brennecke LH, Aranyi C, Heck JD. 13-week inhalation toxicity study of menthol cigarette smoke. Food Chem.Toxicol. 35, 683-692, 1997.
Gaworski CL, Dozier MM, Heck JD, Gerhart JM, Rajendran N, David RM, Brennecke LH, Morrissey R. Toxicologic evaluation of flavor ingredients added to cigarette tobacco: 13-week inhalation exposures in rats. Inhalation Toxicology 10, 357-381, 1998.
[17] Dalbey WE, Nettesheim P, Griesemer R, Caton JE, Guerin MR, Chronic inhalation of cigarette smoke by F344 rats. J.Natl Cancer Inst. 64, 383-390, 1980.
Wehner AP, Dagle GE, Milliman EM, Phelps DW, Carr DB, Decker JR, Filipy RE. Inhalation bioassay of cigarette smoke in rats. Toxicol Appl Pharmacol. 61, 1-17, 1981.
Coggins CR, Ayres PH, Mosberg AT, Ogden MW, Sagartz JW, Hayes AW. Fourteen-day inhalation study in rats, using aged and diluted sidestream smoke from a reference cigarette. I. Inhalation toxicology and histopathology. Fundam Appl Toxicol. 19, 133-140, 1992.
[18] Coggins CR, Lam R, Morgan KT. Chronic inhalation study in rats, using cigarettes containing different amounts of Cytrel tobacco supplement. Toxicology. 22, 287-296, 1981.
[19] OECD, Guideline for testing of chemicals. Test No. 413: Subchronic inhalation toxicity: 90-day study. 2009. Paris, France, Organization for Economic Co-operation and Development.
[20] Vanscheeuwijck PM, Teredesai A, Terpstra PM, Verbeeck J, Kuhl P, Gerstenberg B, Gebel S, Carmines EL. Evaluation of the potential effects of ingredients added to cigarettes. Part 4: subchronic inhalation toxicity. Food and chemical toxicology. 40, 113-131, 2002.

rule (c*t = constant3) applies (Kaegler et al., 2000; Kaegler et al., 2001)[21] also other regimens are feasible.
Test atmosphere exposure conditions should be selected to be within the sensitive (dynamic) range of as many biological endpoints as possible.
The test atmosphere is characterized by performing chemical analyses on relevant markers. For cigarette smoke these would include both particulate and gas vapor phase markers (e.g., TPM, carbon monoxide, nicotine, and selected aldehydes). Particle size distribution should be characterized, when applicable.
Care is taken that the generation, dilution, and transfer of the smoke is identical for the test and control groups in order to assure that effects like redistribution of smoke constituents between gas and particulate phase and precipitation of particulate phase components in the tubing are the presented to the rats.
Exposure of the animals to the test atmosphere should be confirmed by biomonitoring (i.e., respiratory physiology, blood carboxyhemoglobin, blood nicotine/cotinine, and/or urinary nicotine metabolites as appropriate).
Biological activity is assessed by evaluating the following endpoints according to OECD guideline 413 (OECD, 2009)[22]: in-life observation, mortality, body weight development, food consumption, respiratory physiology, ophthalmology, hematology, clinical chemistry, organs weight, gross pathology, histopathology of the respiratory tract, histopathology of the non-respiratory tract organs. In addition, pulmonary inflammation might be further assessed by evaluation of the differential amounts of inflammatory cells in bronchoalveolar lavage fluid.

Result

ROEMER FOR PHILIP MORRIS
A 90-day inhalation study in rats was performed to evaluate the impact of three different ingredient mixes on the in vivo biological activity represented by selected endpoints of mainstream smoke of test kretek cigarettes in comparison to MS from a kretek control cigarette. Overall, the data indicate that the addition of ingredient mixes including d-limonene up to 3 ppm did not increase the biological activity as measured in this study.

2.2.10 Toxicological Assessment

Smoking causes serious disease and is addictive. More than 5,000 chemicals -- or smoke constituents - are formed when tobacco is burned. More than 100 of these smoke constituents have been identified by public health authorities as causes or potential causes of smoking related diseases, including cardiovascular disease (heart disease), lung cancer, and chronic obstructive pulmonary disease (emphysema, chronic bronchitis). Smokers are far more likely to become sick with one of these diseases than non-smokers. In addition, smoking is addictive, and it can be very difficult to stop smoking.

D-limonene has been evaluated in an ingredient mixture according to the toxicological assessment criteria for tobacco products outlined in the General Overview and Principles of Toxicological Assessment of Cigarette Ingredients, Product Designs and Manufacturing Processes, August 2009. Following an evidence-based approach considering the available literature data, the smoke

[21] Kaegler M, Anskeit E, Teredesai A, Vanscheeuwijck P, Terpstra P. Stability of Different Exposure Regimens in Subchronic Inhalation Studies With Cigarette Smoke. The Toxicologist. 60[1 Suppl.], 431-432, 2001.
Kaegler M, Teredesai A, Vanscheeuwijck P, Terpstra P, Gerstenberg B. Comparison of Two Exposure Regimens in Rat Subchronic Inhalation Studies With Cigarette Smoke. The Toxicologist. 54[1 Suppl.], 17, 2000.
[22] OECD, Guideline for testing of chemicals. Test No. 413: Subchronic inhalation toxicity: 90-day study. 2009. Paris, France, Organization for Economic Co-operation and Development.

chemistry data and the biological effects of cigarette smoke as seen with the bacterial mutagenicity assay, the cytotoxicity assay, the mouse lymphoma assay, the in vivo micronucleus assay, and the subchronic inhalation study we have concluded that d-limonene, at the use levels, does not increase the inherent toxicity of tobacco smoke.

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MODULE 3.1

LIST OF REFERENCES

Baker, R.R. et al. An overview of the effects of tobacco ingredients on smoke chemistry and toxicity. Food and Chemical Toxicology (42S), 53-83. 2004.

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Pesonen, M. et al. Occupational contact dermatitis caused by D-limonene. Contact Dermatitis, 71, 273-279. 2015

Piadé, J. J., et al. Toxicological assessment of kretek cigarettes Part 3: Kretek and American-blended cigarettes, inhalation toxicity. Reg Tox Pharmacol 70, Supplement 1: S26-S40. 2014.

Piadé, J. J., et al. Toxicological assessment of kretek cigarettes: Part 2: Kretek and American-blended cigarettes, smoke chemistry and in vitro toxicity. Reg Tox Pharmacol 70, Supplement 1: S15-S25. 2014.

Roemer, E., et al. Toxicological assessment of kretek cigarettes Part 6: The impact of ingredients added to kretek cigarettes on smoke chemistry and in vitro toxicity. Reg Tox Pharmacol 70, Supplement 1: S66-S80. 2014.

Roemer, E., et al. Toxicological assessment of kretek cigarettes: Part 1: Background, assessment approach, and summary of findings. Reg Tox Pharmacol 70, Supplement 1: S2-S14. 2014.

Sanders, E. et al. Does the use of ingredients added to tobacco increase cigarette addictiveness?: a detailed analysis. Inhal. Toxicol. (24), 227-245. 2012.

SCCNFP, Opinion Concerning Fragrance allergy in consumers, A review of the problem, analysis of the need for appropriate consuemr information and identificaiton o fconsumer allergens. 1999

Schramke, H., et al. Toxicological assessment of kretek cigarettes. Part 7: The impact of ingredients added to kretek cigarettes on inhalation toxicity. Reg Tox Pharmacol 70, Supplement 1: S81-S89. 2014.

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MODULE 3.2

PERIODICAL LITERATURE REVIEW REPORT

Literature review is carried out regularly and the relevant publications are included in this document twice per year: i.e. in the first quarter (U1 session) and in the third quarter (U2 session).

2014

Refers to the following columns in the Toxicological Table	Reference	Update session