Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

EC Number: **204-317-7**
CAS Number: **119-36-8**
Index Number: **-**

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Note on confidential information

Please be aware that this report is intended to be made publicly available. Therefore it should not contain any confidential information. Such information should be provided in a separate confidential Annex to this report, clearly marked as such.

CONTENTS

SUPPORT ON HOW TO COMPILE ANNEX I TO THE CLH REPORT

Annex I to the CLH report may be compiled from DARs, CARs and/or other sources. Non-confidential DAR/CAR can be annexed as such provided that it has sufficient level of details on the studies. The DS is encouraged to remove any irrelevant parts of the DAR/CAR. The DS must ensure that Annex I can be published during PC, i.e. it does not contain any confidential information.

For support, below is an example on how each study could be presented individually under its own subchapter including the study reference, detailed study summary and results. The format of the detailed study summary of an individual study is flexible as long as the summary is clearly reported and under a correct hazard class. Detailed support can be found below under each subchapter. If DAR/CAR is annexed to the CLH report as Annex I, it must be indicated clearly in the evaluation part of the report where in Annex I the relevant study can be found. If read-across to structurally or mechanistically similar substance is used please provide a justification for using data from this substance and, if known, present the calculations to convert dose/concentration levels from the test substance to the substance for which CLH is proposed. Please provide also a justification for providing non-testing data by any other approaches such as quantitative structure-activity relationships (QSARs) or grouping methods. Support on grouping of substances and read-across can be found in the following links:

http://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_qsars_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_readacross_en.pdf

http://www.qsartoolbox.org/

http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm

http://echa.europa.eu/en/view-article/-/journal_content/title/assessing-read-across-how-echa-does-it

1 PHYSICAL HAZARDS

Ideally the reported study should comply with the indication provided in ECHA guidance "How to report robust study summaries".

1.1 Explosives

Statement based on the chemical structure of the substance.

1.2 Flammable gases (including chemically unstable gases)

Not applicable (liquid)

1.3 Oxidising gases

Not applicable (liquid)

1.4 Gases under pressure

Not applicable (liquid)

1.5 Flammable liquid

1.5.1 [Study 1]

Study 1 reference:

Handbook data, Merck Index, 14 th edition 2006

Material and methods

- Closed-cup method
- Purity not available

Results

- Flash-point: 99°C (no data on pressure)

1.6 Flammable solids

Not applicable (liquid)

1.7 Self-reactive substances

Not assessed

1.8 Pyrophoric liquids

Not assessed

1.9 Pyrophoric solid

Not applicable (liquid)

1.10 Self-heating substances

1.10.1 [Study 1]

Study 1 reference:

Database GESTIS - Database on Hazardous substance 1999

Material and methods

- No data on the method
- Methyl salicylate, purity not available

Results

- 450°C, no data on pressure

1.11 Substances which in contact with water emit flammable gases

Not assessed

1.12 Oxidising liquids

Statement based on the chemical structure of the substance.

1.13 Oxidising solids

Not applicable (liquid)

1.14 Organic peroxides

Not applicable, the substance does not contain the bivalent O-O structure and is not derivatives of hydrogen peroxide where one or both of the hydrogen atoms have been replaced by organic radicals

1.15 Corrosive to metals

Not assessed

2 HEALTH HAZARDS

2.1 Toxicokinetics

2.1.1 Yamagata *et al.* (1976)

Study reference

Yamagata T, Inoue H, Kodama R et al. 1976a. Metabolic fate of methyl salicylate. I. Absorption, distribution and excretion in hairless mice. Oyo Yakuri 12:371-383.

Method

Mice hairless HRS/J (hr); Single gavage dose of 97 mg/kg (2.62 mg mixture of [14C]MeS & unlabelled MeS, radioactivity 1.25 uCi)

Tissues and body fluids sampled: blood, serum, liver, spleen, kidney, adrenal, thymus, heart, lung, brain, pancreas, ovary, uterus

Time and frequency of sampling: 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 hours after dosing

Whole body autoradiography: 9 mice were anaesthetised with ether at times from 0.25 to 48 hours following administration. They were immediately frozen in an acetone-solid CO2 mixture, embedded in an aqueousCMC. Sagittal sections of the whole body (50um thickness) were prepared and exposed to Xray film for 3 weeks.

Measurement of tissue radioactivity: Blood samples were collected from the femoral artery and vein at the specified time intervals after administration/application. The animals were sacrificed by exsanguination and laparotomised to excise the various organs. [14C]MeS retained in the plaster was extracted with benzene. Tissues were processed and radioactivity measured using a dioxane scintillator in a Tri-carb liquid scintillation spectrometer (Packard model 3375).

Measurement of urinary and faecal excretion: Naturally excreted urine & faeces were collected at the specified times after administration of [14C]MeS. Urine samples were diluted with water. Faeces were extracted with methanol and extracts diluted with water. Radioactivity was measured as for tissue radioactivity.

Results:

Absorption was close to 100%. Blood level of radioactivity reached a maximum at 30 minutes followed by a rapid decrease. Tissue levels of radioactivity reached a maximum at 30 minutes. Radioactivity levels were high in the liver, kidneys and adrenals but low in the lungs, heart, spleen and pancreas, with the lowest level in the brain.

Tissue levels of radioactivity after oral application of [14C]MeS (Tissue levels as d.p.m/mg wet tissue

	0.25	0.5	1	2	4	8	12	24	48
Liver	84.7±7.6	95.6±8.7	84.2±11.2	62.2±14.6	28.2±3.8	7.7±3.2	2.7±0.3	0.9±0.03	0.6±0.1
Spleen	31.9±1.5	32.7±1.0	28.2±3.4	17.5±4.7	5.2±1.1	1.5±0.4	tr	tr	tr
Kidney	115±11.2	131±19.2	92.1±6.9	58.1±12.6	28.9±2.1	11.0±4.5	0.4±0.0	0.5±0.0	0.2±0.0
Adrenal	103±21.5	105±26.0	80.9±18.0	36.9±9.8	23.7±5.1	8.2±3.4	tr	tr	tr
Thymus	24.0±3.0	26.6±5.2	28.3±3.2	14.9±4.4	9.4±3.5	2.8±0.8	1.5±1.1	tr	tr
Heart	49.1±4.3	56.1±9.5	36.0±5.7	24.7±6.4	6.2±2.0	2.0±0.7	tr	tr	tr
Lung	64.5±3.3	79.5±4.6	45.3±4.1	27.7±7.0	8.5±3.2	5.4±1.7	tr	tr	tr
Brain	12.3±0.6	15.7±1.7	9.3±2.9	5.6±2.4	1.7±0.7	0.6±0.1	tr	tr	tr
Pancreas	33.3±1.1	33.3±2.9	27.2±3.7	14.1±3.0	6.2±1.5	1.6±0.4	tr	tr	tr
Ovary	30.3±11.5	42.8±10.4	38.6±4.7	25.2±8.8	13.1±5.9	2.8±0.7	0.6±0.2	tr	tr
Uterus	61.1±7.8	64.5±7.6	55.1±1.7	30.6±1.3	13.2±3.2	5.1±1.8	tr	tr	tr
Serum	217±47.1	237±18.1	166±16.8	134±35.2	37.8±10.0	14.1±5.2	0.7±0.0	0.6±0.0	tr
Blood	102±20.4	112±7.2	81.8±11.9	69.6±17.8	12.9±5.9	5.4±2.2	0.4±0.1	0.3±0.0	tr

tr: trace (< 0.14 d.p.m/mg wet tissue

Recovery of radioactivity after oral application of [14C]MeS (% applied radiation)

	0.25 h	0.5 h	1 h	2 h	4 h	8 h	12 h	24 h	48 h
Tissues	8.22±0.42	9.14±0.44	7.62±0.42	5.35±0.31	1.83±0.10	0.71±0.04	0.16±0.01	0.07±0.00	0.03±0.00
GI tract & contents	64.1±9.10	39.1±6.66	30.2±5.10	15.3±1.93	13.6±2.45	1.80±0.23	0.12±0.00	0.06±0.00	0.05±0.00
Blood	7.00±0.05	7.68±0.49	5.59±0.81	4.76±1.20	0.89±0.41	0.37±0.15	0.03±0.00	0.02±0.00	tr
Urine	-	-	21.7±8.90	38.0±12.1	56.5±8.46	100.0±9.95	96.8±8.48	99.7±8.49	103.0±8.76
Faeces	-	-	0.09±0.04	0.10±0.04	0.32±0.09	0.31±0.05	0.53±0.07	1.09±0.38	0.51±0.15
Carcass	25.3±2.67	43.1±8.31	32.8±6.26	28.2±1.00	26.0±10.0	3.01±0.69	0.21±0.03	0.75±0.24	tr
Total recovered	104.6±5.12	99.0±15.1	98.0±8.82	91.9±11.4	99.1±14.4	106.2±9.35	97.8±9.28	101.7±7.62	103.6±8.70

2.1.2 Davison *et al.* (1961)

Study reference

Davison C, Zimmerman EF, Smith PK. 1961. On the metabolism and toxicity of methyl salicylate. J Pharmacol Exp Ther.;132: 207-11.

Method in animals

10 male rat Wistar exposed by oral gavage to methyl salicylate at 500 mg/kg, calculated as free salicylic acid. Vehicle = methyl cellulose.

Under the same experimental conditions; the oral absorption and metabolism of MeS in rats have been compared with that of NaS and ASA.

Tissues and body fluids sampled: plasma (2 ml) and 4 ml of brain homogenate (one part tissue and three parts water).

Time and frequency of sampling: the blood was removed by intracardic puncture at 20 or at 60 minutes after administration.

Results:

MeS does not produce any higher plasma or brain concentrations than NaS and ASA, and is completely hydrolyzed to free salicylate in as little as 20 minutes.

MeS, NaS, and ASA are all rapidly absorbed; with NaS being the most rapid.

Table 1 presents the mean values obtained for total salicylate in groups of 10 animals. MeS values are not given because they were negligible.

Tissue Analyzed	Minutes after Administration	After MeS (500 mg/kg*)	After NaS (500 mg/kg*)	After ASA (500 mg/kg*)
Plasma	20	217 +/- 16.1(x)	296 +/- 22.3	209 +/- 18.6
Brain	20	8 +/- 2.7	38 +/- 5.9	22 +/- 4.4
Plasma	60	278 +/- 16.7	316 +/- 24.8	274 +/- 23.5
Brain	60	42 +/- 7.3	52 +/- 7.4	38 +/- 5.5

Table 1: Plasma and brain concentrations of total salicylate in mg/l* in rats after the oral administration of theree salicylates.

MeS: methyl salicylate
NaS: Sodium salicylate
ASA: acetysalicylic acid
*: calculated as free salicylic acid
(x): Mean +/- standard error. Each figure is an average of determinations in ten animals.

Method in humans

Absorption of methyl salicylate (MeS) into the human bloodstream was compared with that of acetylsalicylic acid. The chemicals were administered in the morning to six young healthy adults (four men and two women). All subjects had fasted at least 10 hours. 0.42 ml (7 mg/kg as SA) of MeS or ASA were orally administered. Fifteen ml of blood were withdrawn 15 and 90 minutes later and plasma analyses were carried out on all samples.

Results

After 15 min, the mean MeS and free salicylate values were 4.9 and 7.9 mg/l, respectively. After 90 min, these values were 2.8 and 10.5 mg/l, respectively. 30% MeS remained unhydrolysed at 15 minutes, and 21% at 90 minutes.

Table 1: Comparison of plasma salicylate levels in humans in mg/l (dose averaged 7 mg/kg as SA) after oral administration of MeS or ASA

Subject ID	MeS administration						ASA administration	
	15 min			90 min			15 min	90 min
	MeS	Free Sal.	Total Sal.	MeS	Free Sal.	Total Sal.	Total Sal.	Total Sal.
PS	6.4	8.8	15.2	3.2	5.0	8.2		
DC	3.2	7.7	10.9	5.7	21.1	26.8	16.9	26.8
LE	4.7	7.4	12.1	2.5	7.3	9.8	7.3	22.3
LS	4.5	13.6	18.1	0	10.8	10.8	25.0	34.0
CD	4.5	6.0	10.5	1.3	9.6	10.9	16.9	20.4
EZ	6.3	4.4	10.7	4.1	9.1	13.2	25.0	18.4
Mean	4.9	7.9	12.8	2.8	10.5	13.3	18.2	24.5
SD	0.05	1.27	1.26	0.82	2.29	2.78	3.26	2.87

2.1.3 Yano *et al.* (1986)

Study reference

Yano T, Nakagawa A, Tsuji M, Noda K. 1986. Skin permeability of various non-steroidal anti-inflammatory drugs in man. Life Sci.;39(12):1043-50.

Method

28 healthy male volunteers with mean age 29 (18-36) years

Two square areas 1.4x1.4 cm was demarcated by petrolatum on the intact skin of the ventral forearm. 10 µl of acetone solution containing 0.5 mg of the test substance was added dropwise using a microsyringe. The solvent was evaporated by gentle blowing and the area was covered by aluminium foil sealed with surgical tape. The tape was removed together with the foil immediately and 4 hours after application. Following removal, the test substance was recovered from the foil and from the test site. The test substance was determined quantitatively by either UV spectroscopy or GLC. The % absorption was determined by the formula:

%Abs = [1 – (Recovery 4h / Recovery immediate)] x 100

Results

% absorption for SA, MeS and ASA is shown in Table 1. It can be concluded that MeS is almost completely absorbed through human skin.

Table 1

Test Compound	% absorption (0-4 h)	Log P
Salicylic acid	70.8 +/- 2.5	2.25
Methyl salicylate	92.9 +/- 1.8	2.46
Aspirin	16.9 +/- 2	1.23

2.2 Acute toxicity - oral route

2.2.1 Animal data

2.2.1.1 Jenner PM *et al.* (1964)

Study reference:

Jenner PM, Hagan EC, Taylor JM, Cook EL and Fitzhugh. 1964. Food flavourings and compounds of related structure. I. Acute oral toxicity. Fd Cosmet. Toxicol. Vol 2, pp. 327-343.

Detailed study summary and results:

Test type

Groups of 10 young adult Osborne-Mendel rats evenly divided by sex were fasted for approximately 18 hr prior to treatment. Animals had access to water at all times and the food replaced in cages as soon as animals received their respective doses. All doses were given by intubation. All animals were maintained under close observation for recording toxic signs and time of death. Such observation was continued until animals appeared normal and showed weight gain. The usual observation period was 2 weeks; in a few cases, where

no acute toxic signs were seen, the animals were observed for only one week. LD_{50}'s were computed by the method of Litchfield & Wilcoxon (1949).

Study performed prior guideline. Very limited level of details.

Test substance

- Methyl salicylate (no further description)

Test animals

- 10 young adult Osborne-Mendel rats evenly divided by sex (no further information)

Administration/exposure

- Administration by intubation (no further information)

Results and reliability

- Only LD_{50} reported (with 95% confidence limits): 887 mg/kg bw (male/female) (715-110)
- Slope function with 95% confidence limits: 1.5 (1.2-1.8)
- Depression soon after treatment; death time: 4-18 hours

2.2.1.2 Jenner *et al.* (1964)

Study reference:

Jenner PM, Hagan EC, Taylor JM, Cook EL and Fitzhugh. 1964. Food flavourings and compounds of related structure. I. Acute oral toxicity. Fd Cosmet. Toxicol. Vol 2, pp. 327-343.

Detailed study summary and results:

Test type

Groups of guinea-pigs consisting of both males and females were fasted for approximately 18 hr prior to treatment. Animals had access to water at all times and the food replaced in cages as soon as animals received their respective doses. All doses were given by intubation. All animals were maintained under close observation for recording toxic signs and time of death. Such observation was continued until animals appeared normal and showed weight gain. The usual observation period was 2 weeks; in a few cases, where no acute toxic signs were seen, the animals were observed for only one week. LD_{50}'s were computed by the method of Litchfield & Wilcoxon (1949).

Study performed prior guideline. Very limited level of details.

Test substance

- Methyl salicylate (no further description)

Test animals

- Groups of guinea-pigs consisting of both males and females (no further information)

Administration/exposure

- Administration by intubation (no further information)

Results and reliability

- Only LD_{50} reported (with 95% confidence limits): 1060 mg/kg bw (male/female) (873-1300)
- Slope function with 95% confidence limits: 1.6 (1.3-1.9)
- Convulsions. Irritated gastro-intestinal; death-time: 1 hour – 3 days

2.2.1.3 RIFM (1982)

Study reference:

RIFM (Research Institute for Fragrance Materials, Inc.), 1982. Acute toxicity studies. Unpublished report from Givaudan Incorporated, 27 September. Report number 1786 (RIFM, Woodcliff Lake, NJ, USA).

Cited in:

The RIFM Expert Panel. 2007. A toxicologic and dermatologic assessment of salicylates when used as fragrance ingredients. Food and Chemical Toxicology 45:S318–S361

A. Lapczynski, L. Jones, D. McGinty, S.P. Bhatia, C.S. Letizia, A.M. Api. 2007. Fragrance material review on methyl salicylate. Food and Chemical Toxicology 45: S428–S452

Detailed study summary and results:

Test type

The acute oral toxicity of methyl salicylate was determined in Sprague–Dawley rats (5/sex/dose). Methyl salicylate was administered at dose levels of 2.50, 3.15, 3.97, or 5.00 g/kg. Animals were observed for signs of toxicity and mortality over a 14-day period. Gross necropsy was carried out on all animals.

Test substance

- Methyl salicylate (no further description)

Test animals

- Sprague–Dawley rats (5/sex/dose)

Administration/exposure

- Oral, not further specified
- 2.50, 3.15, 3.97, or 5.00 g/kg

Results and reliability

- One male and two females died at 2.50 g/kg; 3/5 males and 4/5 females died at 3.15 g/kg; 4/5 males and 5/5 females died at 3.97 g/kg and all animals died at 5.00 g/kg. Animals died within 48 h of dosing.
- The LD_{50} for males and females was calculated to be 2.82 g/kg (95% CI 2.48–3.21 g/kg); the LD_{50} for males only was calculated to be 3.05 g/kg (95% CI 2.57–3.62 g/kg); and the LD_{50} for females only was calculated to be 2.64 g/kg (95% CI 2.24–3.11 g/kg)
- Clinical signs included piloerection, shaggy coat, hunched posture, lethargy, oscillated movements and difficulty breathing. Necropsy of the animals that died showed severe congestion in the liver, stomach overload, black flakes in the stomach and slight reddening on the mucosal surface of the corpus and antrum of stomach. No significant necropsy findings were noted in the surviving animals.

2.2.1.4 Giroux *et al.* (1954)

Study reference:

Giroux, J., Granger, R., Monnier, P., 1954. Comparative toxicity of methyl diethylacetylsalicylate and methyl salicylate. Société Pharmacie Montpellier 14, 383–390.

Cited in:

A. Lapczynski, L. Jones, D. McGinty, S.P. Bhatia, C.S. Letizia, A.M. Api. Fragrance material review on methyl salicylate. Food and Chemical Toxicology 45 (2007) S428–S452

Detailed study summary and results:

Test type

The acute oral (gavage) toxicity of methyl salicylate was determined in rats. Methyl salicylate was administered in a 20% suspension (w/v) in a gum syrup and water mixture (1:3) at dose levels of 1, 1.25, 1.50, 2, 2.25, 2.50, or 3 g. Clinical signs observed included mydriasis, and convulsions. Necropsy findings included diffuse congestion in the digestive tract and hepatization of the lungs. The LD_{50} was reported to be approximately 1.25 g/kg.

Study performed prior guideline. Very limited level of details reported in the available review from Lapczynski *et al.* (2007).

Test substance

- Methyl salicylate in a 20% suspension (w/v) in a gum syrup and water mixture (1:3) (no further specified)

Test animals

- Rats (no further details)

Administration/exposure

- Oral, not further specified
- 1, 1.25, 1.50, 2, 2.25, 2.50, or 3 g

Results and reliability

- The LD_{50} was reported to be approximately 1.25 g/kg.
- Clinical signs observed included mydriasis, and convulsions. Necropsy findings included diffuse congestion in the digestive tract and hepatization of the lungs.

2.2.1.5 Rumyantsev *et al.* (1992)

Study reference:

Rumyantsev GI, Novikov SM, Andrusov VE, Mel'nikova, Semenovykh. Hygienic standardization of methyl salicylate in workplace air. Gig. Sanit. (1992); 57:28-31

Cited in:

Cosmetic Ingredient Review Panel. 2003. Safety assessment of salicylic acid, butyloctyl salicylate, calcium salicylate, C12-15 alkyl salicylate, capryloyl salicylic acid, hexyldodecyl salicylate, isocetyl salicylate, isodecyl salicylate, magnesium salicylate, MEA-salicylate, ethylhexyl salicylate, potassium salicylate, methyl salicylate, myristyl salicylate, sodium salicylate, TEA-salicylate and tridecyl salicylate. International Journal of Toxicology, 22(Suppl. 3): 1-108.

Detailed study summary and results:

Test type

No information. Publication in Russian; very limited level of details in the publication from the CIR (2003).

Test substance

- Methyl salicylate (not further specified)

Test animals

- Rabbits, guinea pigs, male and female rats, mice (no further information)

Administration/exposure

- Oral (no further details)

Results and reliability

- LD_{50} = 2800 mg/kg bw for rabbits
- LD_{50} = 700 mg/kg bw for guinea pigs
- LD_{50}: 1220 mg/kg bw for male rats
- LD_{50}: 1060 mg/kg bw for female rats
- LD_{50} = 580 mg/kg bw for mice

2.2.1.6 Davison *et al.* (1961)

Study reference:

Davison C, Zimmerman EF, Smith PK. 1961. On the metabolism and toxicity of methyl salicylate. J Pharmacol Exp Ther.;132:207-11.

Detailed study summary and results:

Test type

The oral toxicity of methyl salicylate was evaluated in fasted mice. Because of the insolubility of the ester, the drug was taken up in 2% methyl cellulose at a concentration equivalent to 100 mg/kg of salicylic acid and was given orally. The statistical techniques of Litchfield and Wilcoxon (1949) were used to plot the dose-response curve.

Test substance

- Methyl salicylate (not further specified) in 2% methyl cellulose

Test animals

- Male C3H mice
- 20 to 30 g

Administration/exposure

- Oral (not further specified)
- Statistical method: Litchfield and Wilcoxon (1949)

Results and reliability

- LD_{50}: 1100 mg/kg bw

2.2.1.7 Ohsumi *et al.* (1984)

Study reference:

Ohsumi, T., Kuroki, K., Kimura, T., Murakami, Y., 1984. A study on acute toxicities of essential oils used in endodontic treatment. Journal Kyushu Dental Society 38, 1064–1071

Cited in:

A. Lapczynski, L. Jones, D. McGinty, S.P. Bhatia, C.S. Letizia, A.M. Api. Fragrance material review on methyl salicylate. Food and Chemical Toxicology 45 (2007) S428–S452

Detailed study summary and results:

Test type

The acute oral toxicity of methyl salicylate was determined in ddY male mice (10/dose). Methyl salicylate was administered at dose levels of 1.0, 1.2, 1.3, 1.5, or 1.7 g/kg. Mice were observed for a 7-day period.

Test substance

- Methyl salicylate (not further specified)

Test animals

- ddY male mice (10/dose)

Administration/exposure

- Oral (not further specified)
- 1.0, 1.2, 1.3, 1.5, or 1.7 g/kg
- Observation for 7 days

Results and reliability

- One animal died at 1.0 g/kg; 2/10 died at 1.2 g/kg; 4/10 died at 1.3 and 1.5 g/kg; 9/10 died at 1.7 g/kg. Most animals died on day 1.
- The LD_{50} was calculated to be 1.39 g/kg (95% CI 1.25–1.54 g/kg)

2.3 Skin sensitisation

2.3.1 Animal data

2.3.1.1 Basketter *et al.* (1998)

Study reference:

Basketter DA, Gerberick GF, Kimber I. 1998. Strategies for identifying false positive responses in predictive skin sensitization tests. Food Chem Toxicol.;36(4):327-33.

Detailed study summary and results:

Test type

Review including a LLNA assay. Equivalent or similar to OECD Guideline 429.

Test substance

- Methyl salicylate; no further information (substance tested among other substances in this study)

Test animals

- Groups of four CBA mice

Administration/exposure

- Concentrations tested: 25, 50 and 100%
- Groups of four CBA mice are treated with 25 ml of test material or vehicle alone on the dorsum of both ears. Treatment is performed once daily for three consecutive days. Five days following the initiation of exposure all mice are injected via the tail vein with 250 ml phosphate buffered saline (PBS) containing 20 mCi tritiated thymidine. Mice are killed 5 hr later and the draining lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells is prepared by mechanical disaggregation, washed twice in an excess of PBS, then precipitated with 5% trichloroacetic acid (TCA) at 48C for 18 hr. Pellets are resuspended in TCA and incorporation of tritiated thymidine measured by b-scintillation counting.

Results and discussion

- stimulation index: 0.9 at 25%, 1.0 at 50% and 2.6 at 100%: negative

2.3.1.2 Ashby *et al.* (1995)

Study reference:

Ashby J, Basketter DA, Paton D, Kimber I. 1995. Structure activity relationships in skin sensitization using the murine local lymph node assay. Toxicology.;103(3):177-94.

Detailed study summary and results:

Test type

Serie of LLNA; equivalent or similar to OECD Guideline 429.

Test substance

- Methyl salicylate; no further information (substance tested among 106 substances in this study)

Test animals

- Mice; no further information

Administration/exposure

- Concentrations tested: 5, 10, 25% in Acetone/olive oil 80/20 v/v
- Mice are exposed daily, for 3 consecutive days, to various concentrations of the test chemical or to the relevant vehicle alone, on the dorsum of both ears. Subsequently (conventionally 5 days following the initiation of exposure), mice are injected intravenously with [^{3}H]thymidine and activity measured as a function of isotope incorporation in draining auricular lymph nodes.

Results and discussion

- stimulation index: 0.9 at 5%, 1.4 at 10% and 2.2 at 25%: negative

2.3.1.3 Montelius *et al.* (1998)

Study reference:

Montelius J, Wahlkvist H, Boman A, Wahlberg JE. 1998. Murine local lymph node assay for predictive testing of allergenicity: Two irritants caused significant proliferation. Acta Dermato-Veneriologica; 78:433-437.

Detailed study summary and results:

Test type

LLNA; equivalent or similar to OECD Guideline 429

Test substance

- Methyl salicylate
- CAS 119-36-8

Test animals

- CBA/Ca female mice: 4/dose
- Age at study initiation: 7-10 weeks
- Acclimation period: at least 5 days

Administration/exposure

- Concentrations:
    - Experiment 1: naïve control, DMF control, Methyl salicylate: 10, 20, 25, 50, 100%

- Experiment 2: naïve control, MEK control, Methyl salicylate: 10, 25, 50%
- Experiment 3 (a): naïve control, MEK control, Methyl salicylate: 12.5, 25, 50, 100%
- Experiment 3 (b): naïve control, DMF control, Methyl salicylate: 12.5, 25, 50, 100%
- Vehicle: unchanged or DMF or MEK

- Groups of mice (n=4) received 25µL MeS, neat or dissolved in the vehicle at the concentrations above on the dorsum of both ears daily for 3 consecutive days. Control animals were treated with the vehicle or maintained untreated (naive controls). All mice were injected intravenously 5 days after the first treatment, with 250 µL PBS containing 20 µCi of [3H]thymidine. 5 hours later, the draining auricular lymph nodes were excised and pooled for each group and a single cell suspension of lymph node cells was prepared. Thymidine incorporation was measured with beta-scintillation counting.
- Range-finding tests: not reported

Results and discussion

- There were no deaths and very little sign of irritation (erythema or oedema) at any test sites during the experiment.
- MeS showed a dose-dependent increase in cell proliferation and gave positive results (SI >3) in the LLNA when tested at higher concentrations (50 and 100%). EC_3 values for the four studies were 65, 15, 28 and 33%, with the choice of vehicle having little impact on results.

Treatment	**Concentration MeS (w/v%)**	**Lymph node weight (mg/ node)**	**[^{3}H]Thymidine incorporation (dpm/node)**	**SI relative to vehicle controls**	**SI relative to naïve controls**
Experiment 1					
Naïve	0	2.4	132		-
DMF	0	3.0	368	-	2.8
MeS	10 20 25 50 100	3.5 4.0 4.2 4.6 4.0	454 576 875 945 1464	1.2 1.6 2.4 2.6 4.0	3.4 4.4 6.6 7.2 11.1
Experiment 2					
Naïve	0	2.7	98		-
MEK	0	2.8	148	-	1.5
MeS	10 25 50	3.2 4.0 5.0	264 782 1572	1.8 5.3 10.7	2.7 8.0 16.0
Experiment 3a					
Naïve	0	2.3	150		-
MEK	0	2.9	279	-	1.9
MeS	12.5 25 50 100	3.1 3.2 5.0 4.8	405 465 1697 1984	1.5 1.7 5.9 7.1	2.7 3.1 11.3 13.2
Experiment 3b					
Naïve	0	2.3	150		-
DMF	0	2.3	211	-	1.4
MeS	12.5 25 50 100	3.2 3.9 4.5 4.8	428 509 1607 1984	2.0 2.4 7.6 9.4	2.9 3.4 10.7 13.2

Experiment	Control	EC_3 (%)
1	DMF	65
2	MEK	15
3a	MEK	33
3b	DMF	28

2.3.1.4 Montelius *et al*. (1994)

Study reference:

Montelius J, Wahlkvist H, Boman A. 1994. Experience with the murine local lymph node assay: Inability to discriminate between allergens and irritants. Acta Dermato-Veneriologica; 74:22-27.

Detailed study summary and results:

Test type

LLNA. Equivalent or similar to OECD Guideline 429

Test substance

- Methyl salicylate
- CAS 119-36-8

Test animals

- CBA/Ca female mice: 4/dose
- Age at study initiation: 7-10 weeks
- Acclimation period: at least 5 days

Administration/exposure

- Concentrations:
    - With MEK: 5, 10, 25%
    - With DMF: 1, 5, 25%
- Groups of mice (n=4) received 25µL MeS, neat or dissolved in the vehicle at the concentrations above on the dorsum of both ears daily for 3 consecutive days. Control animals were treated with the vehicle or maintained untreated (naive controls). All mice were injected intravenously 5 days after the first treatment, with 250 µL PBS containing 20 µCi of [3H]thymidine. 5 hours later, the draining auricular lymph nodes were excised and pooled for each group and a single cell suspension of lymph node cells was prepared. Thymidine incorporation was measured with beta-scintillation counting.
- Range-finding tests: not reported

Results and discussion

- MeS did not show a dose-dependent increase in cell proliferation at the lower concentrations, but gave positive results (SI >3) in the LLNA when tested at the concentration of 25%.

Treatment	**Concentration MeS (w/v%)**	**Lymph node weight (mg/ node)**	**[3H]Thymidine incorporation (dpm/node)**	**SI**
Experiment 1				
DMF	0	2.6	228	-
MeS	1 5 25	2.4 3.0 3.4	229 282 676	1.0 1.2 3.0

Experiment 2				
MEK	0	No data	174	-
MeS	5 10 25	No data No data No data	395 428 1301	2.3 2.5 7.5

2.3.1.5 Kimber *et al.* (1998)

Study reference:

Kimber I, Gerberick GF, Basketter D et al. 1998. Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory evaluation. Toxicologist; 42:268.

Detailed study summary and results:

Test type

LLNA. Equivalent or similar to OECD Guideline 429

Test substance

- Methyl salicylate

Test animals

- CBA/Ca (Lab A and B) or CBA/JHsd (Lab C, D, E)
- Age at study initiation: 6-12 weeks
- 5/dose

Administration/exposure

- Concentrations:
  - 1, 2.5, 5.0, 10, 20% in acetone/olive oil (4:1 v/v)
- MeS was tested with 5 other chemicals used as topical medicaments in an interlaboratory evaluation of the LLNA test, in 5 laboratories. Three related protocols were used:
  - Laboratories A (Zeneca) and B (Unilever): Standard LLNA Protocol: Groups of mice (n=5) received 25 µL MeS, dissolved in AOO vehicle at 5 concentrations, or the vehicle alone, on the dorsum of both ears daily for 3 consecutive days. All mice were injected intravenously 5 days after the first treatment, with 250 µL PBS containing 20 µCi of [3H]thymidine. 5 hours later, the draining auricular lymph nodes were excised and pooled for each group and a single cell suspension of lymph node cells was prepared. Thymidine incorporation was measured with beta-scintillation counting.
  - Laboratories C (Proter & Gamble) & D (IITRI): Standard LLNA protocol as above but with lymph nodes pooled for individual mice.
  - Laboratory E (DuPont): Modified LLNA protocol as above, with [^{125}I]iododeoxyuridine in place of ^{3}H-TdR.

Results and discussion

- MeS did not show a significant increase in cell proliferation at any test concentration in any of the three protocols used by the 5 laboratories. In all cases SI <3, with no dose-response.

	Lab A		Lab B		Lab C		Lab D		Lab E	
MeS (%)	dpm	SI	dpm	SI	dpm	SI	dpm	SI	dpm	SI
0 (AOO)	331		374		696		257		64	

1.0	353	1.1	684	1.8	708	1.0	321	1.2	70	1.1
2.5	454	1.4	764	2.0	748	1.1	283	1.1	89	1.4
5.0	469	1.4	578	1.5	1106	1.6	339	1.3	77	1.2
10.0	469	1.4	819	2.2	967	1.4	494	1.9	77	1.2
20.0	660	2.0	655	1.8	954	0.9	311	1.2	55	0.9

2.3.1.6 Gerberick *et al.* (1992)

Study reference:

Gerberick GF, House RV, Fletcher ER, Ryan CA. 1992. Examination of the Local Lymph Node Assay for Use in Contact Sensitization Risk Assessment. Fundam. & Appl. Toxicol.; 19:438-445.

Detailed study summary and results:

Test type

LLNA; equivalent or similar to OECD Guideline 429. Deviations: application of test substance on 4 days with evaluation on day 5.

Test substance

- Methyl salicylate (among 17 chemicals tested in this publication): analytical purity = 90-95%

Test animals

- CBA female mice
- Age at study initiation: 6-9 weeks
- 5/dose

Administration/exposure

- Concentrations:
    - 1, 2.5, 5% in acetone
- Positive control: 1-chloro-2,4,6-trinitrobenzene
- In a slightly midified LLNA protocol, test items were applied on both surfaces of ears of 5 female CBA/J mice per group (25 µl/ear) for four consecutive days (Days 1, 2, 3 and 4). MeS was tested at concentrations of 0, 1, 2.5 and 5% in acetone. On Day 5, the cell proliferation in the local lymph nodes was measured for each individual mouse by incorporation of tritiated methyl thymidine ([^{3}H]TdR). The values obtained were used to calculate stimulation indices (SI). A chemical was considered positive (sensitizer) in this assay if exposure to at least one concentration resulted in a 2-fold or greater increase in [^{3}H]TdR, expressed as disintegrations per minute (dpm) provided that this mean dpm value was statistically different from vehicle-treated mice (p=< 0.01). Chemicals were considered to be moderate to strong sensitizers if the increase was >30-fold and weak to moderate sensitiser if the increase was 2-30-fold over vehicle-treated mice.

Results and discussion

- statistical methods: Intergroup comparisons based on analysis of variance or distribution free methods. Bartlett's test of homogeneity of variance, and least significance difference or Wilcoxon's rank sum test.
- Positive control results: NCB dpm-fold increase: 0.01%: 18.0; 0.05%: 80.3; 0.10%: 103.3
- Methyl salicylate in acetone was considered not to be a sensitizer however the concentrations tested were not high enough to exclude the possibility of sensitization at higher substance concentrations:
    - SI at 1%: 0.8; SI at 2.5%: 0.8; SI at 5%: 0.8 (acetone : SI = 1)

- Disintegration per minute (DPM) : Acetone = 455; MeS 1% = 385; 2.5% = 380; 5% = 380

2.3.1.7 Basketter *et al.* (1992)

Study reference:

Basketter DA, Scholes EW. 1992. Comparison of the local lymph node assay with the guinea pig maximization test for the detection of a range of contact allergens. Food Chem Toxicol; 30:65-69.

Detailed study summary and results:

Test type

LLNA

Test substance

- Methyl salicylate

Test animals

- CBA female mice
- 4/dose

Administration/exposure

- Concentrations:
  - 5, 10, 25% in acetone/olive oil (4:1 v/v)
- Groups of mice (n=4) received 25µL MeS dissolved in the vehicle (AOO) at the concentrations above on the dorsum of both ears daily for 3 consecutive days. All mice were injected intravenously 5 days after the first treatment, with 250 µL PBS containing 20 µCi of [^{3}H]thymidine. 5 hours later, the draining auricular lymph nodes were excised and pooled for each group and a single cell suspension of lymph node cells was prepared. Thymidine incorporation was measured with beta-scintillation counting.

Results and discussion

- MeS showed no increase in cell proliferation and did not give a positive result (SI >3) when tested up to 25%.
  - SI: 5% = 1.3; 10% = 1.0; 25% = 0.8
  - DPM: no data

2.3.1.8 Kimber *et al.* (1991)

Study reference:

Kimber I, Hilton J, Botham PA, Basketter DA, Scholes EW, Miller K, Robbins MC, Harrison PT, Gray TJ, Waite SJ. 1991. The murine local lymph node assay: results of an inter-laboratory trial. Toxicol Lett.;55(2):203-13.

Detailed study summary and results:

Test type

LLNA and maximisation assay

Test substance

- Methyl salicylate among 8 substances tested in the publication
- Sigma Chemical Co., St Louis, MO

- The same batch of test material was used by each of the collaborating laboratories

Test animals

- LLNA: CBA/Ca strain mice 8-12 weeks old; 4/group
- Maximisation test: Dunkin/Hartley strain albino guinea pigs (300-350 g); 9-10/treated group and 4 in the control group

Administration/exposure

- Concentrations
    - 1, 2.5, 5% in AOO for LLNA
    - 2.5% in 0.01% DOBS (dodecyl benzene sulphonate)/saline for intradermal induction and 100% for topical induction.
- LLNA: With minor local variations, the assay was performed in all 4 collaborating laboratories. Groups of mice (n = 4) were exposed on the dorsum of both ears to 25 µl of 1 of 3 concentrations of test chemical or to an equal volume of the relevant vehicle (AOO or DMSO) alone. Treatment was performed daily for 3 consecutive days. Four days following the initiation of treatment all mice were injected intravenously via the tail vein with 250 µl of phosphate-buffered saline (PBS) containing 20 µCi of [^{3}H]methyl thymidine (^{3}H-TdR; specific activity 2 Ci* mmol^{-1}; Amersham International, Amersham, U.K.). Five hours later mice were sacrificed and the draining auricular lymph nodes excised and pooled for each experimental group. A single cell suspension of lymph node cells (LNC) was prepared by gentle mechanical disaggregation through a 200-mesh stainless-steel gauze. Pooled LNC were washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4°C. Approximately 12h later pellets were resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid. Incorporation of ^{3}H-TdR was measured by β-scintillation counting and expressed as mean disintegrations per minute (dpm) per node for each experimental group.
- Maximisation test: Guinea-pig maximization tests were performed by a single laboratory (Laboratory B) according to the method described by Magnusson and Kligman with slight modifications. In preliminary investigations, irritation tests were performed to establish appropriate concentrations of test substances for induction and challenge. Test guinea-pigs (n= 9 or 10) received a series of 6 intradermal injections of the test material at a slightly irritant concentration, in combination with Freund's complete adjuvant, in the shoulder region. Six to 8 days later test animals received, at the same site, a 48-h occluded patch containing the chemical at a mildly irritant concentration. Control guinea-pigs (n=4) were treated with the appropriate vehicle under identical conditions. Twelve to 14 days following the completion of induction both test and control guinea-pigs were challenged on one clipped and razored flank with a 24-h occluded patch containing the test material at the maximum non-irritant concentration. Challenge-induced erythema was determined by visual assessment 24 h later. Maximization tests were performed on the same samples of test material used for the local lymph node assay.

Results and discussion

- grading system used:
    - LLNA: Increases in ^{3}H-TdR incorporation relative to vehicle-treated controls were derived for each experimental group and recorded as stimulation indices.
    - Maximisation test: The results are recorded as the number of animals in the test group responding (exhibiting a positive challenge reaction) together with the mean erythema score (0 = no reaction, 1 = scattered mild redness, 2 = moderate diffuse redness; 3 = intense redness and swelling).
- statistical methods: To evaluate differences between laboratories with respect to (a) derived stimulation indices and (b) the relationship between stimulation indices and test concentrations in the local lymph node assay, an analysis of co-variance was performed for each chemical.

- Results:
    - Maximisation: number of positive response: 0/10; mean erythema score: 0
    - LLNA: negative

Test substance	%	Lab A		Lab B		Lab C		Lab D	
MeS		Dpm ($x10^{-2}$)	SI	Dpm ($x10^{-2}$)	SI	Dpm ($x10^{-2}$)	SI	Dpm ($x10^{-2}$)	SI
	0	2.29	-	3.55	-	1.06	-	3.25	-
	1	2.63	1.1	4.47	1.3	1.90	1.8	3.12	1.0
	2.5	2.34	1.0	3.37	1.0	2.83	2.7	2.37	0.7
	5	2.42	1.1	2.71	0.8	2.71	2.6	3.80	1.2

2.3.1.9 Adenuga *et al.* (2012)

Study reference:

Adenuga D, Woolhiser MR, Gollapudi BB, Boverhof DR. 2012. Differential gene expression responses distinguish contact and respiratory sensitizers and nonsensitizing irritants in the local lymph node assay. Toxicol Sci.;126(2):413-25. doi: 10.1093/toxsci/kfs071.

Detailed study summary and results:

Test type

LLNA

Test substance

- Methyl salicylate
- Purity ≥ 99%; Sigma Chemical (Saint Louis, MO)

Test animals

- BALB/c female mice
- 8-12 weeks of age (Charles River Laboratories)
- 9/group

Administration/exposure

- Concentrations:
    - 20, 40, 80%
    - Dissolved in a 4:1 mixture of acetone and olive oil (4:1 acetone/olive oil)
- The study design followed the standard LLNA dosing regimen (OECD, 2010) and evaluated the dose-dependent responses for the conventional ^{3}HTdR endpoint as well as transcript responses in the draining auricular lymph nodes. Vehicle (4:1 AOO) or one of three concentrations of each test material were applied to the ears (25 µl per ear) of mice (9 mice per group) once daily for three consecutive days (days 1–3) followed by sacrifice on day 6. A group of untreated mice was also included to facilitate examination of vehicle-mediated responses.
- Body weight and erythema scores were evaluated for each mouse on days 1 (prior to first treatment), 2 (erythema scores only), 3, and 6 with auricular lymph node weights recorded at sacrifice. Erythema scores were evaluated to assess the irritant potential of each test material and followed the qualitative scoring outlined in the OECD guideline (OECD, 2010) (0 = no erythema, 1 = very slight, 2 = well defined, 3 = moderate to severe, 4 = severe to eschar). Four mice from each group were

used for ^{3}HTdR determination of the SI, whereas the remaining five mice were used for genomic analysis. For ^{3}HTdR mice, the SI for each group was determined by dividing the mean dpm value for the treatment group by the mean dpm value for the vehicle control group. EC_3 values (concentration that induces a threefold increase in lymphocyte proliferation compared with vehicle control mice) were determined for each chemical by linear (for SI values lying above and below 3) or logarithmic (for SI values higher than 3 for all concentrations) interpolation/extrapolation. To determine the more distal temporal gene expression changes in response to the test materials, an additional time point (study day 10) was included. These mice (5 mice per group) were dermally exposed to vehicle (4:1 AOO) and the high dose only of the test chemical for three consecutive days (days 1–3) followed by sacrifice on day 10. Auricular lymph nodes were only used for gene expression evaluation, and there was no evaluation of stimulation indices via ^{3}HTdR incorporation. However, auricular lymph node weights, body weight, and erythema scores were evaluated for each group.

- RNA extraction: Auricular lymph nodes were excised, weighed, and stored in RNA later. Lymph nodes were homogenized and then further purified by centrifuging followed by total RNA extraction using the RNeasy Mini Kit. Contaminating DNA was removed by on-column DNA digestion with DNase I. Total RNA was quantified using the ND-1000 spectrophotometer and the quality assessed using a 2100 Bioanalyzer. Only samples with a Bioanalyzer RNA integrity number greater than 7 were used for microarray analyses.

- Microarray analysis: Total RNA from mice (3 mice per group) exposed to vehicle or to the highest tested dose of methyl salicylate from day 6 or 10 where amplified and labeled with cyanine 3-labeled CTP (Cy3-CTP). RNA samples with an amplified yield of more than 1.65 µg and a specific activity greater than 9.0 pmol cy3 per µg cRNA were hybridized to an Agilent 4 3 44 K Whole Mouse Genome Oligo microarray slide and then scanned on an Agilent G2565AA Microarray Scanner using the extended dynamic range scanning mode at a 5 lm resolution. Agilent Technologies Feature Extraction software version 9.5.3 was used to extract the data.

- Microarray data normalization, filtering, and statistical analyses: Gene expression data were analyzed using GeneSpring GX 10. Data were normalized. Raw signal values were set to a 5.0 threshold and normalized using a median scaling algorithm (per chip normalization to the 50th percentile value and a per gene normalization to the median value in all samples). Prior to statistical analysis, data were prefiltered on flags (present or absent) and expression levels in order to focus the downstream analysis on the most reproducibly detectable measurements. Statistical analyses employed a parametric ANOVA approach on the dose and/or time variables followed by a Tukey's post hoc test to identify differential gene expression responses relative to vehicle. A false discovery rate of 0.01 was used after applying a Benjamini-Hochberg multiple testing correction. Data clustering and functional annotation were performed using GeneSpring GX 10 software or the Database for Annotation, Visualization and Integrated Discovery (DAVID).

- Quantitative real-time PCR analysis: One microgram of total RNA was reverse transcribed to complementary DNA and used as a template for quantitative real-time PCR (QRT-PCR) utilizing the predesigned TaqMan Gene Expression assay kit on an ABI 7500 Fast Real-Time PCR System. Messenger RNA levels for selected genes from treated samples relative to vehicle controls were determined by the comparative Ct method (DDCT Method) using the housekeeping beta-actin gene as an endogenous control. Statistical analysis was performed on the DCT values for each animal as these values represent the normalized dependent variables from the experimental analysis. The data were tested for equality of variance using Bartlett's test. Dose-response data were subjected to a one-way ANOVA with the factor of dose followed by Dunnett's test. QRT-PCR data from day 10 animals was analyzed using a t-test.

Results and discussion

- There was no alteration in body weight or body weight gain over the course of the study.

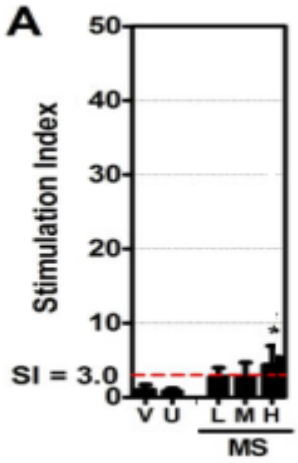
- Erythema scores: very slight to well defined erythema: no excessive irritation

Treatment group	Erythma score			
	Day 2[a]	Day 3[a]	Day 6[a]	Day 10[b]
20% methyl salicylate	0.0	0.6	0.0	-
20% methyl salicylate	0.6	1.0	0.2	-
20% methyl salicylate	0.6	1.6	0.3	0.0

a: Average scores from n = 9 mice.

b: Average scores from n = 5 mice

- Stimulation index: An EC_3 of 48.15% was obtained. Methyl salicylate can be classified as a weak sensitizer according to ECETOC classification.



V: vehicle; U: untreated; MS: methyl salicylate

- Lymph node weights, as an alternative indicator of the lymph node proliferative response, increased dose dependently (statistically significant only with 80% methyl salicylate).
- Microarray data analysis: a total of 188 differentially expressed genes identified with 80% methyl salicylate on day 6 compared with 2 on day 10. The low number of differentially expressed genes at day 10 was consistent with the complete recovery of the responses observed for ear erythema and lymph node weights. A total of 102 gene were differentially expressed in the untreated group relative to the vehicle control on day 6, indicating a possible vehicle effect, which is not unexpected as AOO has been known to elicit variable responses compared to untreated mice.
- Functional evaluation of gene expression profiles:

Functional Categorization of Select Dose-Dependent Changes in Gene Expression on Day 6[a]

Functional category	Common gene name	Gene symbol	Entrez gene ID	UN	MS high
Cell cycle/ proliferation	Antigen Ki-67	*Mki67*	17345	0.65	**1.67**
	Cell division cycle 20	*Cdc20*	107995	0.67	1.36
	Cyclin A2	*Ccna2*	12428	0.59	1.71
	Cyclin B1	*Ccnb1*	268697	**0.58**	**1.71**
	Cyclin E2	*Ccne2*	12448	0.59	1.49
	Cyclin-dependent kinase 1/CDK1	*Cdc2a*	12534	**0.60**	**1.93**
	E2f transcription factor 1	*E2f1*	13555	0.80	1.19
	M-phase inducer phosphatase 3	*Cdc25c*	12532	**0.41**	1.83
Immune response	Aldo-keto reductase family 1, C18	*Akr1c18*	105349	0.62	1.08
	C–C chemokine receptor 4	*Ccr4*	12773	0.91	1.42
	CD5 antigen like	*Cd5l*	11801	1.21	**4.12**
	Chemokine (C–C motif) ligand 6	*Ccl6*	20305	1.45	1.12
	Cluster of differentiation 160	*Cd160*	54215	0.72	1.35
	Cluster of differentiation 207	*Cd207*	246278	0.74	0.73
	Cluster of differentiation 83	*Cd83*	12522	0.76	1.56
	C-type lectin domain family 7	*Clec7a*	56644	1.19	1.15
	Cytotoxic T-lymphocyte antigen 4	*Ctla4*	12477	**0.64**	1.35
	Granzyme B	*Gzmb*	14939	0.58	2.07
	Immunoresponsive gene 1	*Irg1*	16365	1.28	**5.47**
	Interferon gamma	*Ifng*	15978	0.95	1.82
	Interleukin 1 beta	*Il1b*	16176	1.10	0.85
	Interleukin 12 beta subunit	*Il12b*	16160	0.72	0.93
	Interleukin 2 receptor alpha	*Il2ra*	16184	0.77	1.02
	Interleukin 21	*Il21*	60505	0.98	3.30
	Interleukin 4	*Il4*	16189	0.79	**3.11**
	Mast cell protease 1	*Mcpt1*	17224	0.66	0.92
	Mast cell protease 8	*Mcpt8*	17231	0.73	0.77
	Pre-T-cell antigen receptor alpha	*Ptcra*	19208	1.02	2.24
Extracellular matrix	Thrombospondin 1	*Thbs1*	21825	0.80	1.43
	Collagen type 8, alpha 1	*Col8a1*	12837	0.78	2.48
	Matrix metalloproteinase 10	*Mmp10*	17384	0.73	1.42
	Thrombospondin 4	*Thbs4*	21828	0.52	**2.50**
Miscellaneous	Tissue inhibitor of metalloproteinase 1	*Timp1*	21857	1.11	1.33
	Cytoplasmic polyadenylated homeobox	*Cphx*	105594	0.87	1.80
	Fatty acid–binding protein 7	*Fabp7*	12140	0.50	1.25
	FXYD domain–containing ion transport regulator 4	*Fxyd4*	108017	0.60	0.92
	Galectin-7	*Lgals7*	16858	1.31	**3.42**
	Neurotensin	*Nts*	67405	0.60	1.55
	Transient receptor potential cation channel M6	*Trpm6*	225997	1.39	1.73

Note. Bold figures indicate statistical significance relative to vehicle.

UN: untreated; MS: methyl salicylate

Gene Expression Responses for Select Immune-Modulatory Genes on Day 10[a]

Common gene name	Gene symbol	Entrez gene ID	MS
Activation-induced cytidine deaminase	*Aicda*	11628	2.39
Aldo-keto reductase family 1, member C18	*Akr1c18*	105349	0.72
CD5 antigen like	*Cd5l*	11801	1.76
Cluster of differentiation 83	*Cd83*	12522	1.10
Cyclin B1	*Ccnb1*	268697	1.11
Cyclin E2	*Ccne2*	12448	1.30
Fc receptor, IgE, high affinity I, gamma	*Fcer1g*	14127	0.78
Fc receptor, IgE, low affinity II, alpha	*Fcer2a*	14128	0.96
Ig heavy chain (gamma polypeptide)	*Ighg*	380794	0.69
IgM heavy chain	*Igh-6*	16019	1.78
Immunoglobulin lambda chain, variable 1	*Iglv1*	16142	1.00
Interleukin 4	*Il4*	16189	1.52
Secretory leukocyte peptidase inhibitor	*Slpi*	20568	1.32
T-cell Ig and mucin domain–containing 2	*Tim2*	171284	1.50

Note. Bold figures indicate statistical significance relative to vehicle.
[a]Values represent fold changes relative to the vehicle control.

2.3.1.10 Hou *et al.* (2015)

Study reference:

Hou F, Xing C, Li B, Cheng J, Chen W, Zhang M. 2015. Application of BALB/c mouse in the local lymph node assay:BrdU-ELISA for the prediction of the skin sensitizing potential of chemicals. Journal of Pharmacological and Toxicological Methods;Volume 72: 53-58.

Detailed study summary and results:

Test type

LLNA:BrdU-ELISA; conform completely to the procedures and demands in OECD TG 442B (OECD, 2010a) and ICCVAM Recommended Test Method Protocol

Formal validation study conducted to determine if BALB/c mouse could also be used in the LLNA:BrdU-ELISA.

Test substance

- Methyl salicylate
- CAS 119-36-8
- Lot No. 30121828
- Manufacturer: Sinopharm Chemical Reagent Beijing Co., Ltd, Beijing, China

Test animals

- Female BALB/c mice from Beijing HFK Bio-Technology Co., Ltd.
- 8-10 weeks old; 4/group

Administration/exposure

- Concentrations
    - 50% in AOO (acetone: olive oil (4:1))
- The test procedures were performed mainly as the following: 25 μl of the appropriate dilution of the test substance or the vehicle alone was applied to the dorsum of both ears daily for 3 consecutive

days; a single intraperitoneal injection 0.5 ml (5 mg/mouse) of BrdU (10 mg/ml) solution was given on day 5. On day 6, a pair of auricular lymph nodes from each mouse was excised, weighed and stored at -20 °C until BrdU ELISA analysis to measure the level of BrdU incorporation.

- The incorporation of BrdU into lymph node cells (LNC) was measured using a commercial cell proliferation assay kit. First, the bilateral auricular lymph nodes of each animal were crushed, passed through a #70 nylon mesh, and LNC were suspended in 20 ml of physiological saline. Then, 100 µl of the cell suspension was added to the wells of a flat-bottomed microplate in triplicate. Simultaneously, 100 µl of physiological saline was added to the blank wells. After centrifugation (300g, 10 min), 75 µl supernatants were removed from each well by suction and then the cells were dried at 60 °C for about 1 h. Then, 200 µl of FixDenat solution was added to each well, and the plate was allowed to stand for 30 min at room temperature. After removing the FixDenat solution thoroughly by flicking off and tapping, 100 µl of diluted anti-BrdU antibody solution was added to each well and incubated at room temperature for 90 min. Antibody conjugates were removed by flicking off and rinsing the wells three times with washing buffer. After removing the washing buffer by tapping, 100 µl of the substrate solution was added. Absorbance (ABS) at 370 nm with a reference wavelength of 492 nm was determined with an EPOCH microplate reader at 10, 15, and 20 min later. The optimal result achieving a mean absorbance of the VC group within 0.1–0.2 was used.

Results and discussion

- BrdU labelling index is defined as

BrdU labelling index = $(ABS_{370}$ - ABS $blank_{370}) – (ABS_{492}$ - ABS $blank_{492})$ Results for each treatment group are expressed as the mean stimulation index (SI). The SI is derived from dividing the mean BrdU labelling index/mouse within each test substance group by the mean BrdU labelling index for the solvent/VC group. The average SI for the VCs is one. A positive response is defined as the mean SI of the test group ≥1.6.

- The accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity values and negative predictivity values in this validation study were compared with that in the ICCVAM formal validation study for the LLNA:BrdU-ELISA using CBA/JN mouse (ICCVAM, 2010d). Chi-square test was used with $P > 0.05$ as the level of significant difference. Statistical Product and Service Solutions (SPSS) 19.0 was used for the statistical test.

- Results: negative
    - ICCVAM results Evaluation Report (ICCVAM, 2010b; ICCVAM, 2010e): LLNA:BrdU-ELISA in CBA/JN mice: mean SI for 50% methyl salicylate: 1.44/1.40/1.43
    - Study result with BALB/c mice: mean SI for 50% methyl salicylate: 1.5

Comparison of the performance of LLNA:BrdU-ELISA using BALB/c mice with that of using CBA/JN mice.

Comparison	*n*	Accuracy		Sensitivity		Specificity		False positive rate		False negative rate		Positive predictivity value		Negative predictivity value	
		%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
LLNA:BrdU-ELISA in BALB/c mice	42	90.5	38/42	96.8	30/31	72.7	8/11	27.3	3/11	3.2	1/31	90.9	30/33	88.9	8/9
LLNA:BrdU-ELISA in CBA/JN mice [a]	43	95.3	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	32/34	100	9/9

Chi-square test, $P > 0.05$. No statistically significant difference exists between LLNA:BrdU-ELISA using BALB/c mouse and LLNA:BrdU-ELISA using CBA/JN mouse regarding the accuracy, sensitivity, specificity, false positive rate, false negative rate, positive predictivity values and negative predictivity values.

[a] , Data from ICCVAM LLNA:BrdU-ELISA Evaluation Report (ICCVAM, 2010d).

2.3.1.11 Klecak *et al.* (1977)

Study reference:

Klecak G, Geleick H, Frey JR. 1977. Screening of fragarance materials for allerginicity in the guinea pig. Journal of the Society of Cosmetic Chemists; Vol. 28(2): 53-64.

Detailed study summary and results:

Test type

Open epicutaneous test (OET), Draize test, maximisation test and Freund's complete adjuvant test (FCAT)

OET, Draize test and FCAT: no EU harmonized guideline

Maximisation test: deviation from OECD Guideline 406: few number of animals used

Test substance

- Methyl salicylate among 32 compounds tested in this study

Test animals

- Male and female outbred Himalayan white-spotted guinea pigs
- Weight: 400-500 g
- 6-8 animals/group

Administration/exposure

- OET: The compounds were tested undiluted as well as dissolved in acetone, ethanol, diethyl phthalates etc at concentrations of 30, 10, 3, 1, 0.3, 0.1, and 0.03% (or less when necessary). Before the induction procedure, the skin irritation caused by a single application was determined. For this purpose, 0.025 ml of each undiluted compounds and its progressively diluted solutions was applied to an area measuring 2 cm^2 previously marked with a circular stamp on the clipped flank skin of 6 to 8 animals per group. In each case, the liquid tested was applied uniformly with a pipette. After evaporation of the solvent, the application site was left uncovered. The reactions were read after 24 h using an "all or none" criterion, i.e. the dose-response curve was established by endpoint determination. The minimal irritating concentration was defined as the lowest concentration causing mild erythema in at least 25% of the animals of the group. The highest concentration of a compound used in this local application test was determined by its solubility and skin irritating capacity. The determination of the tolerance threshold on the guinea pigs in the OET is mainly done for methodical reasons, in order to quantitatively realize an eventual sensitisation and besides it gives information on the concentration-dependent skin tolerance of substances. *Induction procedure:* on day 0, 0.1 ml of each undiluted compound and its progressively diluted solutions were applied to an area measuring 8 cm^2 on the clipped flank skin of 6-8 guinea pigs per concentration group, using 4 to 6 such groups for each compound. The applications were repeated daily for 21 days, always using the same skin site. The application site was left uncovered and the reactions were read 24 hours after each application. The maximum nonirritant and the minimal irritating concentrations were determined by the same "all or none" criterion. When necrotis or ulcerating reactions were provoked, the application site was changed. *Challenge procedure*: all groups of guinea pigs previously treated for 21 days as well as 6 to 8 untreated controls for each compound were tested on days 21 and 35 on the contralateral flank with the same compound at the minimal irritating concentration and at some lower nonirritant concentration. These tests were performed by applying with a pipette 0.025 ml of each concentration to skin areas measuring 2 cm^2, the reactions being read after 24, 48 and/or 72h. A concentration was considered allergenic when at least 2 out the 8 animals of the concentration group concerned showed positive reactions with non irritant concentrations used for challenge.
- Draize test: a dose of 0.05 ml of a 0.1% solution of the compound tested in isotonic saline was injected intradermally on day 0 and further doses of 0.1 ml each were injected on 9 alternate days (total dose = 0.95 mg). The treated and untreated animals were challenged intradermally with 0.05 ml of a 0.1% solution on days 35 and 49. The evaluation criterion was the mean diameter of the popular reactions.
- Maximisation test: on day 0 the animals were injected intradermally with 0.1 ml of a 5% solution of the compound tested with 0.1 ml of a 5% emulsion of the same compound in Freund's complete adjuvant (FCA) and with 0.1 ml of FCA alone, each injection being given twice. In addition 250 mg of the compound dissolved in petroleum at a concentration of 25% was applied on day 8 to a clipped skin area of the neck and was kept under occlusive bandage for 2 days. On day 21 an occlusive patch

test with the compound at a subirritant concentration in petroleum was applied to the flank for 24 hours. The reactions were read 24 and 48 h after removing the patch.

- FCAT: doses of 0.05 ml of the undiluted compounds mixed with the same volume of FCA were injected intradermally into the neck on days 0, 2, 4, 7 and 9. The control animals were similarly treated with 5x0.05 ml of FCA alone. All the animals were tested epicutaneously on days 21 and 35.

Results and discussion

- OET: Positive
    - Minimal irritating concentration after 1 application: 3%; after 21 applications: 3%
    - Minimum sensitizing concentration: 30%; minimum eliciting concentration: 1%
    - No relationship was found between skin irritation and the capacity to induce contact hypersensitivity
- Draize test: negative
- Maximisation test: negative
- FCAT: negative

2.3.1.12 Arts *et al.* (1997)

Study reference:

Arts JHE, Droge SCM, Spanhaak S et al. 1997. Local lymph node activation and IgE responses in Brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals. Toxicology; 117:229-237.

Detailed study summary and results:

Test type

LLNA and IgE measurement

IgE measurement: no EU harmonized guideline

LLNA: not followed OECD guidelines. The LLNA was performed in rats instead of mice and BrdU was used instead of tritiated thymidine but protocol not followed the OECD guideline 442B.

Test substance

- Methyl salicylate
- Purity: at least 99% (Sigma USA)

Test animals

- Female Wistar and Brown Norway rats

Administration/exposure

- Concentations
    - 5, 12.5, 25% in acetone/olive oil (4:1 v/v) for LLNA
    - 25% on day 0 and 12.5% on day 7 for IgE measurement
- The LLNA procedure was similar to the standard assay for mice, however bromodeoxyuridine (BrdU) was used instead of tritiated thymidine. Ear-draining lymph nodes were fixed in 70% ethanol and sectionned. following treatment with monoclonal antiBrdU antibody, biotin-labelled rabbit-anti-mouse antibody, peroxidase-conjugated strepavidin and chromogen 3,3'diaminobenzidine

tetrahydrochloride, mitotic activity was determined. 50 cells were examined to determine the mean percentage of BrdU positive cells per compartment and the weighted average percentage of BrdU+ cells per section. As a measure of total proliferation, the average percentage of BrdU+ cells per section was multiplied by lympb node weight.

- For serum IgE responses, animals received 150 µL of the selected concentrations of test substance on each flank (approximately 12 cm^2 each). 7 days after the first application, all animals received 75 µL of the same chemical at 50% of the initial concentration on the dorsum of both ears. The IgE test was performed with the highest concentration tested in the LLNA. Blood was sampled by orbital puncture on days 8 and 14 and from the abdominal aorta at sacrifice on day 21. Serum IgE was measured in individual or pooled serum by ELISA. The concentration of IgE in test serum samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as ug IgE/mL serum.

Results and discussion

- LLNA: Negative
    - SI: 5% = 0.8; 12.5% = 0.4; 25% =0.8 in Wistar rats
    - SI: 5% = 1.0; 12.5% = 1.0; 25% = 1.2 in Brown rats
- IgE: no increase in serum IgE concentration
    - 0.47 +/- 0.06 µg/ml on day 8; 0.73 +/- 0.31 on day 14; 0.68 +/- 0.26 on day 21

2.3.1.13 Picotti *et al*. (2006)

Study reference:

Piccotti JR, Knight SA, Gillhouse K, Lagattuta MS, Bleavins MR. 2006. Evaluation of an *ex vivo* murine local lymph node assay: multiple endpoint comparison. J Appl Toxicol.;26(4):333-40.

Detailed study summary and results:

Test type

Ex vivo LLNA: no OECD guideline available

In vivo LLNA: equivalent to OECD 429 guideline

Test substance

- Methyl salicylate
- Purity: 99.7% (Sigma USA)

Test animals

- Female BALB/c mice
- 8-9 weeks old
- 4-6/group

Administration/exposure

- Concentations: 25% in acetone
- Mice were treated by topical application with 25 µl of methyl salicylate (25% v:v) in acetone to the dorsum of both ears on days 1–3 using a single channel pipetter. Control animals were treated with vehicle (acetone) alone. Animals were euthanized on day 6 by carbon dioxide inhalation.
- *Ex vivo* LLNA: At scheduled necropsy, the auricular lymph nodes draining the ears were removed, weighed and placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U ml-1 penicillin, 0.1 mg ml-1 streptomycin,

10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 0.56 mM L-arginine, 14 µM folic acid, 0.27 mM asparagine, 1.5 mM L-glutamine and 50 µM 2-mercaptoethanol. The lymph nodes from individual animals were processed into single-cell suspensions, and cell numbers determined on an Abbott Cell-Dyne® 3500 hematology analyser using the auxiliary mode. Lymph node cell density was adjusted to 1×10^6 cells ml^{-1}, and 200 µl of the cell suspensions were then added to a 96-well U-bottom plate. Cells were incubated with 0.5 µCi/well of [methyl-3H]-thymidine for 16-18 h at 37 °C/10% CO_2. Thymidine incorporation into DNA was measured using a Beckman LS6500 liquid scintillation counter. Data are reported as disintegrations per minute (dpm)/animal and stimulation index (SI). A test substance that induced a SI of ≥ 3 in the *ex vivo* LLNA was considered a positive finding.

- *In vitro* BrdU incorporation: Following cell isolation, lymph node cells were adjusted to 1×10^6 cells ml^{-1}, and 3 ml of the cell suspensions were added to 6-well tissue culture plates. Cells were incubated at 37 °C/10% CO_2 for 1 h with 10 µM BrdU. After the incubation period, the cells were fixed with 3 ml of 70% ethanol, and processed for cell cycle analysis. Briefly, nuclei were isolated and incubated on ice for 30 min with an anti-BrdU-FITC antibody. Following a wash step, 500 µl of a 50 µg ml-1 propidium iodide (PI) solution and 25 µl of 600 Kunitz units ml-1 RNase A were added, and samples were incubated in a 37 °C water bath for 15 min in the dark. Counterstaining with PI allowed for visualization of varying DNA content. Samples were analysed using a Becton Dickinson FacScan flow cytometer. The FITC signal was detected at approximately 520 nm, and propidium iodide fluorescence at approximately 620 nm. The percentages of cells in Sphase (DNA synthesis) of the cell cycle are reported.
- *In vivo* LLNA: On day 6, mice were given a single 0.25 ml intravenous injection of [methyl-^{3}H]-thymidine (80 µCi ml^{-1}) into a tail vein, resulting in a total dose of 20 µCi of radioisotope per mouse. The draining lymph nodes of the ears were removed after approximately 5 h, placed in phosphate buffered-saline (PBS), and then processed into single cell suspensions. Cells were washed and resuspended in 5% trichloroacetic acid (TCA) to precipitate macromolecules. The samples were stored overnight at 2–8 °C. Thymidine incorporation into DNA was measured using a Beckman LS6500 liquid scintillation counter. Data are reported as dpm/animal and SI.

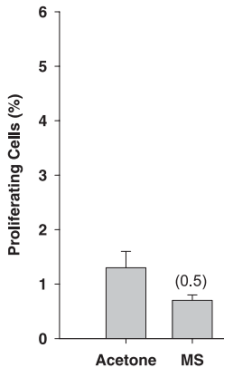
Results and discussion

- *Ex vivo* LLNA: Negative (SI = 1.5) with methyl salicylate at 25%
- Lymp node weight and cellularity: no effect on lymph node weight or cell number

Table 2. Evaluation of lymph node weight and cellularity

Treatment group	Lymph node		Cellularity	
	Weight (mg)	SI	Cell no. (10^6)	SI
Acetone	6.6 ± 1.3	–	6.7 ± 1.1	–
Methyl salicylate, 25%	5.6 ± 0.7	0.9	7.2 ± 0.5	1.1

- Assessment of *ex vivo* BrdU Incorporation as a Marker for Lymphocyte Proliferation:



- Comparison *ex-vivo* LLNA and Standard LLNA :
    - *Ex-vivo* LLNA (initial experiment repeated) : SI = 3 (positive) with methyl salicylate at 25%; no increase in lymph node weight or cellularity
    - *In vivo* LLNA: SI = 2.5 with methyl salicylate at 25%
    - No skin irritation (i.e erythema) seen

Table 3. Comparison of an *in vivo* and *ex vivo* LLNA

Treatment group	*In vivo*		*Ex vivo*	
	dpm	SI	dpm	SI
Acetone	82 ± 16	–	371 ± 46	–
Methyl salicylate, 25%	203 ± 8	2.5	1087 ± 69[a]	3.0

2.3.2 Human data

2.3.2.1 Lapczynski *et al.* (2007a)

Study reference:

Lapczynski A, Jones L, McGinty D, Bhatia SP, Letizia CS, Api AM. 2007. Fragrance material review on methyl salicylate. Food Chem Toxicol.;45 (1):S428-52. Epub 2007 Sep 14.

Review report.

Original study: Research Institute for Fragrance Material, Inc., 1976a. Report on human maximization studies. RIFM report number 1796, July 23 (RIFM, Woodcliff Lake, NJ, USA. Unpublished data.

Detailed study summary and results:

Test type

A human maximization test was conducted on 25 healthy volunteers. Wintergreen oil (containing 80–99% methyl salicylate) in petrolatum was applied under occlusion, to the same site on the volar forearms of 25 subjects for 5 alternate-day 48-h periods. The patch sites were pretreated for 24 h with 5% aqueous SLS under occlusion for the initial patch only. Following a 10–14-day rest period, a challenge patch of methyl salicylate was applied to a fresh site for 48 h under occlusion. Prior to the challenge, 5% SLS was applied to the test sites for 30 min under occlusion on the left side of the back, while methyl salicylate was applied without SLS treatment on the right side. Additional SLS controls were placed on the left and petrolatum on the right, and labeled as the fifth site. No reactions were observed with 12% wintergreen oil

2.3.2.2 Lapczynski *et al.* (2007b)

Study reference:

Lapczynski A, Jones L, McGinty D, Bhatia SP, Letizia CS, Api AM. 2007. Fragrance material review on methyl salicylate. Food Chem Toxicol.;45 (1):S428-52. Epub 2007 Sep 14.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials, Inc., 1973b. Report on human maximization studies. RIFM report number 1803, November 26 (RIFM, Woodcliff Lake, NJ, USA). Unpublished data.

Detailed study summary and results:

Test type

A maximization test was carried out on 27 healthy volunteers using 8% methyl salicylate in petrolatum. An occluded patch with methyl salicylate was applied to the same sites of the forearms of each subject for five alternate 48-h periods. Patch sites were pre- treated with 5% aqueous SLS under occlusion. Following a 10–14-day rest period a challenge patch was applied to a fresh site for 48 h under occlusion. An application of 10% aqueous solution of SLS under occlusion was applied 1 h prior to challenge. Reactions were read at patch removal and 24 h later. No sensitization reactions were observed

2.3.2.3 Lapczynski *et al*. (2007c)

Study reference:

Lapczynski A, Jones L, McGinty D, Bhatia SP, Letizia CS, Api AM. 2007. Fragrance material review on methyl salicylate. Food Chem Toxicol.;45 (1):S428-52. Epub 2007 Sep 14.

Review report.

Original study: RIFM (Research Institute for Fragrance Materials, Inc., 1964. Repeated insult patch test of methyl salicylate in human subjects. Unpublished report from IFF Incorporated, 3 April. Report number 12617 (RIFM, Woodcliff Lake, NJ, USA). Unpublished data.

Detailed study summary and results:

Test type

A human repeated insult patch test (HRIPT) was conducted in 39 male and female volunteers (13 male/26 female) with 1.25% methyl salicylate. A 0.5 ml aliquot of methyl salicylate (vehicle not provided) was applied to a 1-inch square Webril patch fixed to the center of 1 x 3 in. strip of adhesive elastic bandage and placed on the upper arm of each subject. Patches were removed 24 h later. A total of nine applications were made over a three week period. The patches were applied to the same sites unless a reaction was observed. A challenge patch was applied to a fresh site on the Monday of the sixth week and removed 24 h later. Reactions were scored at 24 and 72 h after patch removal. No sensitization was observed.

2.3.2.4 Romaguera & Grimalt. (1980)

Study reference:

Romaguera C, Grimalt F. 1980. Statistical and comparative study of 4600 patients tested in Barcelona (1973-1977). Contact Dermatitis.;6(5):309-15.

Detailed study summary and results:

Test type

A total of 4600 patients were patch tested in the 5-year period 1973-1977 in the Allergy Department of Barcelona University; 106 patients failed to return for the reading of patches, so are not included. The patients studied have been divided into 4 well-defined groups:

1) 2784 patients (60.52%) diagnosed as contact dermatitis from the clinical features and results of patch testing
2) 189 patients (4.11%) considered to be cumulative insult dermatitis of the hands caused by primary irritants at home
3) 135 patients (2.94%) with photoallergic and phototoxic reactions.
4) 1491 patients (32.43%) with negative patch tests results

The standard tests comprise the allergens recommended by ICDRG with the exception of some allergen mixtures tested separately. Methyl salicylate was tested at 2% in petroleum.

Results: 6/4600 patients with positive result (0.13%)

2.3.2.5 Rudner (1977)

Study reference:

Rudner EJ. 1977. North American Group results. Contact Dermatitis.;3(4):208-9.

Detailed study summary and results:

Test type

Patch test of the North American Contact Dermatitis Group from 1 July 1975 to 30 June 1976. The Al-Test® and Dermicel® tape were used. Tests were read at 48 and 96h. A total of 900-2000 patients were tested in the 1975-76 series. Not all patients received the complete “battery” of patch tests.

Results: 183 patients tested with methyl salicylate 2% with 1.6% of reactivity.

2.3.2.6 Ferguson & Sharma (1984)

Study reference:

Ferguson J, Sharma S. 1984. Cinnamic aldehyde test concentrations. [Letter to the Editor]. Contact Dermatitis; 10:191–192.

Detailed study summary and results:

Test type

Patch tests from October 1981 and June 1983 in Scotland: 241 consecutive patients (180 females and 61 males) tested for their sensitivity to fragrances in a perfume screening series, including 2% methyl salicylate in PMF.

Results: 3 females with positive reactions (grade 2 [definitive erythema] and above); no male with positive reaction. Incidence of positive reactions: 1.2%.

2.3.2.7 Mitchell (1982)

Study reference:

Mitchell JC, Adams RM, Glendenning WE, Fisher A, Kanof N, Larsen W, Maibach HI, Rudner EJ, Schnorr W, Storrs F, Taylor JS. 1982. Results of standard patch tests with substances abandoned. Contact Dermatitis; 8:336–337.

Detailed study summary and results:

Test type

Results of standard patch tests on eczema patients in North America. Methyl salicylate included as 2% in petroleum:

- 301 patients included between 1978-1979 : 1% positive reaction
- 284 patients included between 1979-1980 : 2% positive reactions

2.3.2.8 Nethercott *et al.* (1989)

Study reference:

Nethercott JR, Nield G, Holness DL. 1989. A review of 79 cases of eyelid dermatitis. Journal of the American Academy of Dermatology; 21:223–230.

Detailed study summary and results:

Test type

Between January 1980 and May 1987, 1091 patients were assessed and given patch tests in the Contact Dermatitis Clinic of St Michael's Hospital. The scoring method recommended by the International Contact Dermatits Research Group was used for scoring test results. The patch test chemicals were supplied by Trolle-Larsen, Copenhagen, Denmark and Hermal-Chemie, Reinbek, West Germany. Nineteen patients with eyelid dermatitis and 70 patients with dermatitis at other sites were tested with the perfume series recommended by Larsen, including methyl salicylate 1% in petroleum. Chemicals were applied to Al-Test strips or Finn chambers, which were then secured to the upper aspect of the back with Scanpor tape. Patch tests were applied on Mondays, Wednesdays or Fridays and were first read on the 2 days after the tests were applied. When the chambers or strips were removed at the first reading (at 48 or 72 hours), sites were scored 30 to 60 minutes after the appliance had been removed to allow adequate time for the physical effects of the pressure of the appliance on the skin to resolve. The second reading was scored at 48 or 72 hours after the first reading. One observer scored all the responses to patch tests. Responses scored as 1+, 2+ or 3+ were determined to be positive; doubtful responses were scored as negative. Irritant responses were scored as negative.

Results:

- Patients with eyelid dermatitis: 0% positive reaction
- Patients with dermatitis at other sites: 1.4% positive reactions

2.3.2.9 de Groot *et al.* (2000)

Study reference:

de Groot AC, Coenraads PJ, Bruynzeel DP, Jagtman BA, van Ginkel CJW, Noz K, van der Valk PGM, Pavel S, Vink J, Weyland JW. 2000. Routine patch testing with fragrance chemicals in The Netherlands. Contact Dermatitis; 42: 184–185.

Detailed study summary and results:

Test type

In a multicentre study, 9 fragrance allergens, including methyl salicylate 2% in petroleum, were tested in all patients routinely patch tested. Test procedures were carried out according to internationally accepted criteria. Test concentrations were chosen on the basis of published data and potential irritancy was excluded in a pilot study involving 200 patients.

From September 1998 to April 1999, 1825 patients were tested. Seven patients (0.4%) were positive to methyl salicylate. Among them, 4 (0.2%) were negative to the Fragrance mix.

2.3.2.10 Addo *et al.* (1982)

Study reference:

Addo HA, Ferguson J, Johnson BE, Frain-Bell W. 1982. The relationship between exposure to fragrance materials and persistent light reaction in the photosensitivity dermatitis with actinic reticuloid syndrome. British Journal of Dermatology;107:261–274.

Detailed study summary and results:

Test type

A group of 50 PD/AR (photosensitivity dermatitis with actinic reticuloid) subjects was studied to determine the incidence of contact allergic sensitivity to a range of common fragrance materials. Thirty-two examples of polymorphic light eruption (PLE), nine of whom gave a positive clinical history of intolerance to perfume materials, and 457 consecutive cases of contact dermatitis (CD) routinely attending the contact dermatitis clinic for patch testing to the European standard series of allergens, were studied for comparison. Fragrance materials used for patch testing included methyl salicylate 2% in PMF.

Each subject was patch-tested to the various fragrance materials using a standard closed patch test technique. Approximately equal amounts (10 mg) of the materials as supplied in yellow soft paraffin (PMF) were applied to standard Al-test patches which were then placed on the skin of the upper back and held in place with Scanpor adhesive tape. The patches were removed at 48 h and any reactions read at that time, and at 72 h after the application were recorded using the following grades of response: o = no response; 1/2 = doubtful; 1 = faint but definite erythema; 2 = definite erythema; 3 = erythema + oedema; 4 = erythema + oedema + vesicles.

Results:

- 1 positive reaction in PD/AR subjects
- 0 positive reaction in PLE and CD subjects

Methyl salicylate was also tested *in vitro* to determine its phototoxicity. The first was the simple microbiological test described by Daniels (1965) but using *Candida utilis* rather than the pathogenic *Candida albicans* (Kagan, Gabriel & Reed, 1980) freshly seeded on Sabouraud's dextrose agar plates. Filter paper discs, saturated with fragrance material, or patch test materials in PMF, were placed on the surface of duplicate plates, one set being exposed to UV-A from a battery of four fiuorescent tubes (Thorn: Ultraviolet/non filter 2Q W; irradiance approximately 12 mW/cm2) for 48 h and the other set kept in the dark. Phototoxicity is demonstrated in this test by clear zones around the materials in the irradiated plates while there is complete growth in those kept in the dark. Where there was no evidence of phototoxicity with UV-A, an alternative test was used in which a limited exposure to UV-B was given. This consisted ofa 15-min exposure (approximately 1350 mj/cm^2) to the emission from a battery of four Westinghouse FS20 sunlamp fiuorescent tubes (irradiance approximately 15 mW/cm^2) which did not kill the yeast, but by which a phototoxic effect of this wavelength region could be demonstrated. The second test used washed human red blood cells (RBCs) suspended in barbitone buffered saline, pH 7.4, at a dilution of I: 500 (Frain-Bell *et al*, 1979). A 1 ml amount of the material to be tested, at various dilutions in ethyl alcohol, was added to 99 ml of the RBC suspension. Aliquots of 5 ml, forming cell monolayers in petri dishes, were exposed to UV-A or UV-B from batteries of fluorescent tubes as in the first method, but for limited times up to 3 h. After the exposure the dishes were kept in the dark for 30 min and then the suspetisions were centrifuged, the released haemoglobin in the supernatant being determined as cyanmethaemoglobin. The method used is an adaptation of that using Drabkins' solution of i g sodium bicarbonate, 0.2 g potassium ferricyanide and 0.05 g potassium cyanide in 1 l of distilled water. An addition of 2 ml of this solution was made to 2 ml of the supernatant, the mixture was left for at least 30 min and the resulting colour was read at 420 nm in a spectrophotometer against a blank of Drabkins' solution and buffered saline. The degree of photohaemolysis may be expressed as a percent of that obtained with RBCs diluted in distilled water. Controls were run using ethyl alcohol alone and the fragrance materials without exposure to radiation.

Results: negative at 10% in the Candida UV-A test and in the photohaemolysis test with UV-A and UV-B at 0.1%

2.3.2.11 Stingeni *et al.* (1995)

Study reference:

Stingeni L, Lapomarda V, Lisi P. 1995. Occupational hand dermatitis in hospital environments. Contact Dermatitis; 33:172–176.

Detailed study summary and results:

Test type

A self-administered questionnaire to identify subjects with hand dermatitis was distributed to 1532 employees of the Perugia Monteluce Hospital in Italy. The clinical examination comprised a standardized interview, a clinical evaluation and skin tests (patch and/or prick tests). These were planned only for subjects with anamnestic hand eczema and/or atopic mucosal reactions (total of 276 patients with occupational contct dermatitis: 82 males and 194 females). Patch testing was performed with the GIRDCA standard series (FIRMA Diagent, Florence, Italy), our "health" series and, when necessary, a "rubber" series. The last 2 series included chemicals present in the trade-marked products most commonly employed in our hospital, including methyl salicylate 2% in petroleum. All allergens were applied on the back with Van der Bend square chambers and removed after 2 days. Readings were carried out at 2 and 3 days and reactions considered positive when erythema, infiltration, papules and/or vesicles occurred inside and outside the test site. The diagnosis of hand contact dermatitis was established on objective signs at the time of examination, the history and the results of skin tests.

Results: only 14/276 health operators reacts positively to the allergens tested. No positive reaction reported with methyl salicylate.

2.3.2.12 Oiso *et al*. (2004)

Study reference:

Oiso N, Fukai K, Ishii M. 2004. Allergic contact dermatitis due to methyl salicylate in a compress. Contact Dermatitis.;51(1):34-5.

Detailed study summary and results:

Test type

A 79 year-old woman had a rectangular pruritic erythematous macule on the hip following the use of a Teipap A Cool® compress. The manufacturer provided samples of the methyl salicylate and other ingredients that are used to manufacture this compress. Patch testing showed a positive reaction to methyl salicylate 2% o.o on D2(+) and D3(+).

2.3.2.13 Hindson (1977)

Study reference:

Hindson C. 1977. Contact eczema from methyl salicylate reproduced by oral aspirin (acetyl salicylic acid). Contact Dermatitis.;3(6):348-9.

Detailed study summary and results:

Test type

A 63 year-old Iraqi businessman was being treated with paracetamol 250 mg thrice daily for pain due to osteoarthritis of the cervical spine and was referred when he developed an acute dermatitis of the neck, upper back, shoulders and dorsa of the hands. The patient had been applying an analgesic ointment to his neck and back for 6 weeks in addition to prescribed therapy. The ointment contained menthol, camphor and 12% methyl salicylate but no antihistamines or local anaesthetics. Patch testing to the constituents of the ointment

(2% in olive oil) gave a positive reaction to methyl salicylate at 48h, still present at 72h and negative reactions to the other ingredients.

He was advised to avoid all proprietary applications and the rash subsided with topical steroid therapy while he continued to take paracetamol as before. He returned to Germany and Iraq and 3 months later returned to the UK with a reappearance of his eczema at the previous sites, although he denied using any topical applications except a steroid cream for a few days prior to appointment. It transpired that he had subsequently received treatment from physicians in Germany and Iraq because of his neck pain and had ceased paracetamol therapy. It was not possible to identify his Iraqi and German tablets completely but analysis showed that both of them contained aspirin (acetyl salicylic acid). A non-aspirin containing analgesic was substituted and further patch testing carried out with results as follows:

Aspirin aqueous 0.3 % : negative at 48h

Aspirin suspension aqueous 5 % : negative at 48h

Sodium salicylate 2 % aqueous : ++ve at 48h

Methyl salicylate 2 % in arachis oil : ++ve at 48h

Negative results to the above were found in three control patients. Five days later with his consent he was given 500 mg of aspirin and noticed pruritus and erythema again in the previously affected areas.

2.4 Reproductive toxicity

2.4.1 Animal data

2.4.1.1 FDA (2006a)

Study reference:

FDA. 2006. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029. December 13, 2006

Detailed study summary and results:

Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

Test substance

- Methyl salicylate
- Lot Y096
- 100.1%

Test animals

- Rats/Crj:CD(SD)IGS
- 20/sex/group for main study + 3/sex/group for toxicokinetics (satellite groups)

Administration/exposure

- 30, 100, 300 mg/kg/day. The high dose was based on the 2-week repeated dose preliminary study showing a depressive trend in body weight gain in males receiving 300 mg/kg and a decrease in food consumption in males and females receiving 300 mg/kg. The middle and low doses were set in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepated for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within 100 ± 5%)
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily from 2 weeks prior to mating through the mating period and up to day 6 of gestation for the females beginning at 10 weeks of age. The administration period before copulation in the males was determined as 2 weeks, since methyl salicylate did not show any testicular toxicity in the 2-week repeated dose preliminary study (dosage: 30, 100, 300 mg/kg).

Description of test design:

- Females aged 12 weeks were housed overnight with males aged 12 weeks in a 1:1 ratio. Copulation was confirmed by the presence of a vaginal plug or sperm in the vaginal smear on the following morning and the day of confirmed copulation was designed as day 0 of gestation. Mating was conducted within the same group for a maximum of 2 weeks. As for pairs for which copulation was unsuccessful, the males were mated with non-treated females and the females were mated with successfully copulating males in the same group during a maximum of 1 week.
- Parameters: clinical observation (clinical signs and mortality twice daily during administration period and once daily during other periods), body weight and food consumption (twice weekly and daily for females during gestation), estrous cycle (every morning), reproductive ability test (days required for successful copulation, copulation index, male and female fertility indices), necropsy (testes, left epididymis, ovaries, uterus and skin of the treated site) and organ weight (testes and epididymides), sperm (at necropsy, sperm count, sperm motility, sperm form anomalies index), numbers of corporea lutean implants, dead and live embryos, pre-implant loss index, dead embryo index.
- Determination of plasma salicylic acid concentration: blood was collected 1 time at 4 hours after administration on day 0 (first day) and 13 (final day) of administration. The concentration of salicylic acid was measured by HPLC.

Results and discussion

- Statistical analysis: mean and standard deviations with regard to body weight, body weight gain, food consumption, organ weight, estrous cycle, count of estrus, number of days required for successful copulation, sperm count and numbers of corporea lutea, implnats and live embryos were calculated for every group and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The copulation index and male and female fertility indices were analysed by χ^2 test. The sperm motility, sperm form anomalies index, pre-implant loss index and dead embryo index by Wilcoxon's rank sum test. Levels of $P < 0.01$ and $P < 0.05$ were considered to be significant in all cases.

Effects on parent animals

- One male in the 300 mg/kg group showed hypoactivity, bradypnea, hypothermia and blanching on day 3 of administration and died on day 4 of administration. In addition, crust on the treated site

and/or loss of hair were observed in 2 females in the 300 mg/kg group from day 9 of administration to day 13 of gestation. There were no mortality or abnormal signs in the males and females in the control, 30 and 100 mg/kg groups.

- A significant lower body weight as compared with the control group was observed throughout the administration period in the males in the 300 mg/kg group and on days 1-3 and 10-14 of administration and throughout the gestation period in the females in the 300 mg/kg group.
- A significant depression of body weight gain as compared with the control group was observed on day 1-49 of administration in the males in the 300 mg/kg group (≥ -20%) and on days 10-14 of administration in the females in the 300 mg/kg group. During the gestation period, a significant depression of body weight gain as compared with the control group was observed on days 5 and 9-13 of gestation in the females in the 300 mg/kg group.

		Mean Body Weight Gain (g) ± SD in Males in the High Dose Group	
Day of Treatment	**Vehicle Control**	**300 mg/kg/day**	**% Change of Control**
1	5.3 ± 3.7	-6.3 ± 7.7**	↓219%
3	17.4 ± 4.7	-3.1 ± 19.8**	↓117.8.0%
7	40.0 ± 7.9	16.1 ± 13.4**	59.8%
10	55.4 ± 11.0	29.2 ± 16.0**	47.3%
14	74.7 ± 13.1	47.8 ± 16.5**	36.0%
17	83.4 ± 14.0	52.9± 18.7**	36.6%
21	97.7 ± 16.7	68.6 ± 19.9**	29.8%
24	114.5 ± 17.3	83.4± 21.9**	27.2%
28	126.3 ± 95.3	95.3 ± 24.9**	24.5%
31	140.7 ± 20.6	107.8 ± 26.1**	23.4%
35	153.4 ± 21.7	120.5 ± 30.8**	21.0%
38	163.3 ± 24.0	126.6 ± 31.9**	22.5%
42	174.9 ± 24.0	137.4 ± 32.1**	21.4%
45	182.3 ± 24.1	145.5 ± 34.9**	20.2%
49	194.1 ± 24.2	154.9 ± 37.0*	20.2%

** Significantly different from vehicle control ($p<0.01$)

Table 8 Body weight gains in female rats

Group and dose		Vehicle control			30 mg/kg			100 mg/kg			300 mg/kg		
							Body weight gain (g)						
Days of treatment	1	0.3±	6.3	(20)	-2.8±	5.6	(20)	-5.2±	6.1*	(20)	-7.8±	7.1**	(20)
	3	4.5±	8.3	(20)	4.4±	5.5	(20)	3.0±	5.2	(20)	-4.9±	12.8*	(20)
	7	13.8±	8.0	(20)	14.3±	6.4	(20)	10.8±	6.6	(20)	11.2±	7.1	(20)
	10	22.7±	9.8	(20)	20.8±	8.9	(20)	16.9±	8.2	(20)	13.8±	11.6*	(20)
	14	28.8±	10.9	(20)	28.9±	10.3	(20)	23.8±	9.2	(20)	19.0±	12.2*	(20)

*: P<0.05, **: P<0.01 (significantly different from vehicle control).
Values are mean±S.D. and the values in parentheses represent the number of animals.

Table 9 Body weight gains in Fo dams

Group and dose		Vehicle control			30 mg/kg			100 mg/kg			300 mg/kg		
							Body weight gain (g)						
Days of gestation	1	6.7±	4.9	(20)	8.9±	2.5	(18)	6.7±	4.1	(19)	7.9±	3.5	(19)
	2	12.8±	4.0	(20)	15.1±	4.2	(18)	13.7±	4.0	(19)	13.3±	4.8	(19)
	3	19.1±	4.7	(20)	19.9±	3.9	(18)	19.6±	5.1	(19)	19.3±	6.3	(19)
	4	24.2±	4.6	(20)	26.5±	4.8	(18)	24.1±	6.2	(19)	22.9±	6.7	(19)
	5	29.9±	6.4	(20)	31.2±	4.8	(18)	27.9±	6.7	(19)	24.8±	6.3*	(19)
	6	34.9±	6.7	(20)	34.5±	6.3	(18)	32.4±	6.3	(19)	31.5±	5.6	(19)
	7	40.2±	6.6	(20)	42.3±	6.8	(18)	37.4±	6.9	(19)	35.9±	7.6	(19)
	8	45.5±	6.6	(20)	47.0±	6.4	(18)	43.5±	8.1	(19)	41.7±	7.2	(19)
	9	51.7±	8.3	(20)	51.8±	7.0	(18)	46.9±	8.3	(19)	44.4±	7.3*	(19)
	10	56.9±	8.5	(20)	56.9±	8.5	(18)	51.2±	9.2	(19)	50.1±	8.3*	(19)
	11	63.4±	9.5	(20)	64.1±	7.6	(18)	58.3±	8.7	(19)	54.5±	8.7**	(19)
	12	68.1±	10.0	(20)	69.3±	8.2	(18)	62.0±	9.9	(19)	59.7±	8.9*	(19)
	13	74.3±	9.4	(20)	74.3±	9.0	(18)	68.3±	10.2	(19)	66.2±	8.2*	(19)

*: P<0.05, **: P<0.01 (significantly different from vehicle control).
Values are mean±S.D. and the values in parentheses represent the number of dams.
Four animals (30 mg/kg:2, 100 mg/kg:1 and 300 mg/kg:1) were non-pregnant.

- A significant decrease in food consumption as compared with the control group was observed in the 300 mg/kg group: in males on day 1 of administration (-27%) and in females on days 1 (-28.7%) and 3 (-17.1%) of administration and days 4 and 5 of gestation (-9 and -11%). Significant increase of food consumption as compared with the control group was observed on day 7 of administration in

the 300 mg/kg group but this increase was considered to be incidental change not related to methyl salicylate administration since the change was transient.

- In necropsy of the males, retention of an oily fluid in the subcutis of the treated site was observed in all the males in the control and methyl salicylate groups. Dark red spot in the thymus and dark red macule in the subcutis of the treated site was observed in the dead male in the 300 mg/kg group. In necropsy of the dams on day 13 of gestation, retention of an oily fluid in the subcutis of the treated site was observed in all the dams in the control and methyl salicylate groups and in the non-pregnant females in the 30, 100 and 300 mg/kg groups. Crust of the treated site or loss of hair was observed in 2 dams each in the 300 mg/kg group.
- No significant differences in the weights of the testes or epididymides were observed in the methyl salicylate groups as compared with the control group.
- The were no significant differences between the control and methyl salicylate groups in the count of estrus or estrous cycle.

Mean Count of Estrous ± S.D.	3.70 ± 0.47	3.70 ± 0.73	3.90 ± 0.31	3.70 ± 0.80
Mean Estrus Cycle Length ± S.D.	4.10 ± 0.24	4.0 ± 0.0	4.13 ± 0.56	4.46 ± 1.83

- At the first mating, copulation was not confirmed in 1 pair each in the 100 and 300 mg/kg groups and 2, 1, 1 animals in the 30, 100 and 300 mg/kg groups, respectively, were sterile after copulation. Accordingly, the copulation indices were 100, 100, 95.00 and 94.74% for control, 30, 100, 300 mg/kg groups, respectively, and the male and female fertility indices were 100, 90.00, 94.74 and 94.44% for the control, 30, 100 and 300 mg/kg groups, respectively, results showing no significant difference between the control and methyl salicylate groups. No significant differences were observed between the numbers of days required for copulation by the control and methyl salicylate groups. No significant differences were observed between the numbers of days required for copulation by the control and methyl salicylate groups. When one male each in the 100 and 300 mg/kg groups in which copulation was not observed were mated with non-treated females, fertility was confirmed in both cases. When one female each in the 100 and 300 mg/kg groups in which copulation was not observed and in 1 female in the 300 mg/kg group whose paired male had died were mated with males in the same group that were confirmed to have copulated, fertility was confirmed in all cases.
- There were no significant differences between the control and methyl salicylate groups in the sperm form anomalies index, sperm count or sperm motility.

Dose (mg/kg/day)	**№ Examined**	**Spermatogenic Endpoints (Mean ± S.D.)**		
		Count of Sperm ($x10^6$/g)	**Sperm Motility (%)**	**Sperm Form Anomalies Index (%)**
0	20	583.71 ± 149.75	92.24 ± 21.69	7.25 ± 19.94
30	20	632.62 ± 128.30	97.22 ± 2.04	3.97 ± 7.76
100	20	615.37 ± 120.48	97.86 ± 2.07	2.40 ± 2.30
300	19	559.60 ± 184.64	87.62 ± 29.78	10.04 ± 9.02

Effect on early embryonic development:

- A significant decrease in the number of corporea lutea as compared with the control group was observed in the 100 mg/kg group. This change was not dose-related and therefore does not appear to be biologically relevant. There were no significant differences between the control and methyl salicylate groups in the numbers of implants or live embryos, pre-implant loss index or dead embryo index.

Dose (mg/kg)	# Pregnant/Total (%)	(Total №) MEAN (± S.D.)		№ (% of the corpora lutea)
		Corpora Lutea	Implantation Site	Pre-implantation loss
0	20/20 (100%)	312 (15.60 ± 1.60)	297 (14.85 ± 1.57)	15(4.81%)
30	18/20 (90%)	264 (14.67 ± 2.93)	254 (14.11 ± 2.93)	10 (3.79%)
100	18/20 (94.74%)	272 (14.32 ± 1.49)*	267 (14.05 ± 1.54)	5 (1.84%)
300	17/19 (89.5%)	277 (14.58 ± 1.54)	268 (14.11± 1.52)	9 (3.25%)

*: Significantly different from control group ($p<0.05$)

Effects of methyl salicylate on litter (i.e., number of viable ad embryos).

Dose (mg/kg)	№ of Viable Embryos (Mean ± S.D.)	№ of Dead Embryos (% of the # implants)
0	279 (13.95 ± 1.70)	18 (4.81)
30	239 (13.28 ± 2.82)	15 (5.91)
100	253 (13.32 ± 1.77)	14 (5.24)
300	260 (13.68 ±1.57)	8 (2.99)

Plasmatic concentration of salicylic acid

The plasma salicylic acid concentration at 4 hours after administration on day 0 of administration in the 30, 100 and 300 mg/kg groups were 46.4, 147 and 239 µg/ml for the males and 53.5, 164 and 277µg/ml for the females, respectively. The plasma salicylic acid concentration at 4 hours after administration on day 13 of administration in the 30, 100 and 300 mg/kg groups were 46.1, 126 and 290 µg/ml for the males and 47.7, 144 and 300 µg/ml for the females, respectively. These results showed a dose-dependent increase of plasma concentration without clear differences between males and females.

2.4.1.2 Collins *et al.* (1971)

Study reference:

Collins T.F.X, Hansen W.H, Keeler HV. Effect of methyl salicylate on rat reproduction. Toxicology and applied pharmacology 18, 755-765 (1971)

Detailed study summary and results:

Test type

3 generation study

Test substance

- Methyl salicylate
- Source: Dodge and Olcott, Inc., New York (0302655151)

Test animals

- Osborne-Mendel rats
- 20/sex/group

Administration/exposure

- Oral (feed)
- 0, 500, 1500, 3000, 5000 ppm
- The diet was prepared every 14 days

Description of test design:

- The animals fed methyl salicylate at the respective doses for 100 days, after which the animals were mated.
- Parameters assessed in parents: fertility index (number of litters cast/number of females exposed to mating)
- After the birth of the first litter (F1a) observations were made of the number of stillborn and liveborn young and of grossly visible abnormalities. Litters were similarly observed on day 4 and counts were made of the number and conditions of the living pups.When litters exceeded 10 at day 4, the number of pups were reduced to 10. At weaning the animals were sacrificed. The parents (F0) were remated and the same observations were made on the second litter (F1b). At weaning, 20 littermated pairs were selected at each dose level to produce the next generation. The same procedure was followed for succeeding generations except that animals of the third generation were killed and autopsied.
- Supplemental study: in order to test the efficacy of calcium in alleviating or reversing any adverse effect of methyl salicylate, a separate group of F2b rats from each dose level were fed 1500 ppm calcium carbonate in addition to methyl salicylate. The animals were mated and their first and second litters were observed as previously described. Of the 1500 ppm calcium carbonate fed, approximately 600 ppm was available as calcium.

Results and discussion

- A 2-sided chi-square test was used to determine significant differences between each dose and control for fertility index and average litter size. No statistical tests were made on the viability, survival or weaning indexes.
- There was no statistically significant difference ($P < 0.05$) for the fertility index at any level. Appreciable decreases can be seen, however, in the second generation matings at the 5000 ppm level.

TABLE 1

FERTILITY INDEXES OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)									
		0		500		1500		3000		5000	
Generation	Mating	FI[a]	%[b]	FI	%	FI	%	FI	%	FI	%
1	1	20/20	100	20/20	100	20/20	100	20/20	100	20/20	100
	2	19/19	100	20/20	100	18/19	95	19/19	100	20/20	100
2	1	20/20	100	19/20	95	20/20	100	19/20	95	17/20	85
	2	19/19	100	19/20	95	19/19	100	19/20	95	10/13	77
3	1	20/20	100	18/20	90	18/19	95	19/20	95	17/19	89
	2	18/20	90	16/18	89	17/19	89	15/17	88	16/19	84
1, 2, 3[c]	1	60/60	100	57/60	95	58/59	98	58/60	97	54/59	92
	2	56/58	97	55/58	95	54/57	95	53/56	95	46/52	88
3(+Ca)[d]	1	20/20	100	19/20	95	19/20	95	17/20	85	5/5	100
	2	19/20	95	16/20	80	16/20	80	13/20	65	5/5	100

[a] Fertility index (number of litters cast/number of females exposed to mating).
[b] Percent females pregnant.
[c] Not analyzed for statistical significance.
[d] Calcium carbonate (1500 ppm) added to diet.

[3] Source of calcium carbonate: Fisher Scientific, Fairlawn, New Jersey (763830).

- Significant decreases in average litter size was found in the second generation in the second mating at 3000 ppm ($P < 0.05$) and in both matings at 5000 ppm ($P < 0.01$). Altough decreases were seen at 1500 ppm, they were not statistically significant because of the large variation in progeny between females within a group. A dose-related decrease in average litter size per female exposed was apparent in both matings of the second generation starting at 1500 ppm. When the results were combined for all generations, mating 1 and mating 2, there was a clear dose-related decrease starting

at 1500 ppm. When the animals were fed methyl salicylate plus calcium carbonate, significant decreases appeared in the first mating at 3000 ppm ($P < 0.01$) and 5000 ppm ($P < 0.05$).

TABLE 2

AVERAGE LITTER SIZE OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)									
		0		500		1500		3000		5000	
Generation	Mating	No.[a]	Av.[b]	No.	Av.	No.	Av.	No.	Av.	No.	Av.
1	1	208/20	10.4	211/19	11.1	207/20	10.4	235/20	11.8	188/18	10.4
	2	213/19	11.2	232/20	11.6	228/19	12.0	238/19	12.5	198/19	10.4
2	1	216/20	10.8	205/20	10.2	206/20	10.3	169/20	8.4	124/20	6.2[c]
	2	226/19	11.9	204/20	10.2	189/18	10.5	187/20	9.4[d]	86/13	6.6[c]
3	1	192/20	9.6	188/19	9.9	172/19	9.1	170/20	8.5	179/19	9.4
	2	197/20	9.8	191/18	10.6	163/19	8.6	132/17	7.8	172/19	9.1
1, 2, 3[e]	1	616/60	10.3	604/58	10.4	585/59	9.9	574/60	9.6	491/57	8.6
	2	636/58	11.0	627/58	10.8	580/56	10.4	557/56	9.9	456/51	8.9
3(+Ca)[f]	1	201/20	10.0	188/20	9.4	173/20	8.6	130/20	6.5[c]	35/5	7.0[d]
	2	181/20	9.0	179/20	9.0	148/20	7.4	127/20	6.4	43/5	8.6

[a] Total number progeny/number females exposed to mating.
[b] Average litter size per female exposed to mating.
[c] Significant at $P < 0.01$.
[d] Significant at $P < 0.05$.
[e] Not analyzed for statistical significance.
[f] Calcium carbonate (1500 ppm) added to diet.

- Statistically significant differences were observed in the average number of progeny both matings of the second generation at 3000 ppm ($P < 0.05$) and at 5000 ppm ($P < 0.01$). As with average litter size, average liveborn progeny for both matings of the second generation show a consistent dose-related decrease. When the data from all generations are combined for mating 1 and mating 2, there is a consistent decrease with increased dose level starting at 1500 ppm. In the animals fed methyl salicylate and calcium, the average number of liveborn progeny per female exposed showed statistically significant decreases in the first mating at the 3 dose levels from 1500 to 5000 ppm and in the second mating at 3000 ppm. Statistically significant decreases of viability, survival and weaning index were noted in the second generation from 3000 ppm.

TABLE 3

VIABILITY DATA FOR RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)														
		0			500			1500			3000			5000		
Generation	Mating	No.[a]	Av.[b]	VI[c,d]	No.	Av.	VI	No.	Av.	VI	No.	Av.	VI	No.	Av.	VI
1	1	208/20	10.4	1.00	211/19	11.1	1.00	195/20	9.8	0.94	229/20	11.4	0.97	167/18	9.3	0.88
	2	213/19	11.2	1.00	231/20	11.6	1.00	226/19	11.9	0.99	237/19	12.5	1.00	189/19	9.9	0.95
2	1	215/20	10.8	1.00	203/20	10.2	0.99	203/20	10.2	0.99	164/20	8.2[e]	0.97	106/19	5.6[f]	0.85
	2	225/19	11.8	1.00	203/20	10.2	1.00	189/18	10.5	1.00	182/20	9.1[e]	0.97	82/13	6.3[f]	0.95
3	1	188/20	9.4	0.98	184/19	9.7	0.98	160/19	8.4	0.93	164/20	8.2	0.96	174/19	9.2	0.97
	2	196/20	9.8	1.00	186/18	10.3	0.97	155/19	8.2	0.95	118/17	6.9	0.89	166/19	8.7	0.97
1, 2, 3[d]	1	611/60	10.2	0.99	598/58	10.3	0.99	558/59	9.5	0.95	557/60	9.3	0.97	447/56	8.0	0.91
	2	634/58	10.9	1.00	620/58	10.7	0.99	570/56	10.2	0.98	537/56	9.6	0.96	437/51	8.6	0.96
3(+Ca)[g]	1	199/20	10.0	0.99	186/20	9.3	0.99	160/20	8.0[e]	0.92	123/20	6.2[f]	0.95	35/5	7.0[e]	1.00
	2	176/20	8.8	0.97	175/20	8.8	0.98	146/20	7.3	0.99	126/20	6.3[e]	0.99	42/5	8.4	0.98

[a] Total number liveborn/number females exposed to mating.
[b] Average number liveborn per female exposed to mating.
[c] Viability index (no. liveborn/total no. born).
[d] Not analyzed for statistical significance.
[e] Significant at $P < 0.05$.
[f] Significant at $P < 0.01$.
[g] Calcium carbonate (1500 ppm) added to diet.

TABLE 4

SURVIVAL DATA OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)														
		0			500			1500			3000			5000		
Generation	Mating	No.[a]	Av.[b]	SI[c,d]	No.	Av.	SI	No.	Av.	SI	No.	Av.	SI	No.	Av.	SI
1	1	157/17	9.2	0.90	116/14	8.3	0.82	172/19	9.1	0.96	152/15	10.1	0.92	129/15	8.6	0.94
	2	202/19	10.6	0.95	196/20	9.8	0.85	205/19	10.8	0.91	218/19	11.5	0.92	168/19	8.8	0.89
2	1	188/20	9.4	0.87	179/20	9.0	0.88	190/20	9.5	0.94	123/20	6.2[e]	0.75	82/19	4.3[f]	0.77
	2	211/19	11.1	0.94	188/20	9.4	0.93	186/18	10.3	0.98	165/20	8.2[e]	0.91	61/13	4.7[f]	0.74
3	1	174/20	8.7	0.93	177/19	9.3	0.96	147/19	7.7	0.92	139/20	7.0	0.85	147/19	7.7	0.84
	2	174/20	8.7	0.89	179/18	9.9	0.96	150/19	7.9	0.97	113/17	6.6	0.96	153/19	8.1	0.92
1, 2, 3[d]	1	519/57	9.1	0.90	472/53	8.9	0.89	509/58	8.8	0.94	414/55	7.5	0.84	358/53	6.8	0.86
	2	587/58	10.1	0.93	563/58	9.7	0.91	541/56	9.7	0.95	496/56	8.9	0.92	382/51	7.5	0.87
3(+Ca)[g]	1	184/20	9.2	0.92	181/20	9.0	0.97	143/20	7.2	0.89	114/20	5.7[f]	0.93	33/5	6.6	0.94
	2	139/20	7.0	0.79	162/20	8.1	0.92	132/20	6.6	0.90	106/20	5.3	0.84	37/5	7.4	0.88

[a] Total no. day 4 survivors/no. females exposed to mating.
[b] Average no. day 4 survivors per female exposed to mating.
[c] Survival index (no. day 4 survivors/no. liveborn).
[d] Not analyzed for statistical significance.
[e] Significant at $p < 0.05$.
[f] Significant at $p < 0.01$.
[g] Calcium carbonate (1500 ppm) added to diet.

WEANING DATA OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)														
		0			500			1500			3000			5000		
Generation	Mating	No.[a]	Av.[b]	WI[c,d]	No.	Av.	WI	No.	Av.	WI	No.	Av.	WI	No.	Av.	WI
1	1	154/17	9.1	0.98	114/14	8.1	0.98	172/19	9.1	1.00	151/15	10.1	0.99	129/15	8.6	1.00
	2	183/19	9.6	0.91	187/20	9.4	0.95	203/19	10.7	0.99	191/19	10.1	0.88	164/19	8.6	0.98
2	1	176/20	8.8	0.94	168/20	8.4	0.94	188/20	9.4	0.99	121/20	6.0[e]	0.98	74/19	3.9[f]	0.90
	2	200/19	10.5	0.95	173/20	8.6	0.92	179/18	9.9	0.96	160/20	8.0	0.97	48/13	3.7[f]	0.79
3	1	170/20	8.5	0.98	172/19	9.1	0.97	146/19	7.7	0.99	122/20	6.1	0.88	137/19	7.2	0.93
	2	170/20	8.5	0.97	179/18	9.9	1.00	149/19	7.8	0.99	111/17	6.5	0.98	144/19	7.6	0.94
1, 2, 3[d]	1	500/57	8.8	0.96	454/53	8.6	0.96	506/58	8.7	0.99	394/55	7.2	0.95	340/53	6.4	0.95
	2	553/58	9.5	0.94	539/58	9.3	0.96	531/56	9.5	0.98	462/56	8.2	0.93	356/51	7.0	0.93
3(+Ca)[g]	1	183/20	9.2	0.99	181/20	9.0	1.00	140/20	7.0[e]	0.98	108/20	5.4[f]	0.94	33/5	6.6	1.00
	2	121/20	6.0	0.87	144/20	7.2	0.89	130/20	6.5	0.98	102/20	5.1	0.96	28/5	5.6	0.76

[a] Total no. of adjusted day 21 survivors/no. females exposed. Adjusted day 21 survivors = (no. alive at day 21)/(no. kept at day 4) × no. alive at day 4.
[b] Average no. of adjusted day 21 survivors per female exposed to mating.
[c] Weaning index (no. of adjusted day 21 survivors/no. alive at day 4).
[d] Not analyzed for statistical significance.
[e] Significant at $p < 0.05$.
[f] Significant at $p < 0.01$.
[g] Calcium carbonate (1500 ppm) added to diet.

- Decreases in weight at weanling appeared consistently from 3000 ppm.

WEANLING WEIGHTS BY SEX OF RATS FED METHYL SALICYLATE FOR 3 GENERATIONS

		Dietary level (ppm)									
		0		500		1500		3000		5000	
Generation	Mating	M	F	M	F	M	F	M	F	M	F
1	1	40.1[a]	41.3	40.7	38.8	38.0	37.0	32.9	32.6	34.8	33.9
	2	47.0	45.2	45.6	43.0	45.4	42.6	42.1	41.2	37.1	37.5
2	1	46.9	43.6	40.1	39.4	41.1	40.2	40.2	39.4	37.4	34.9
	2	49.0	47.2	43.4	41.0	43.3	42.4	44.6	40.7	42.5	40.2
3	1	46.2	45.5	43.1	41.9	42.8	40.6	42.7	41.9	34.6	31.5
	2	46.3	42.6	46.2	44.6	47.3	46.0	44.7	41.7	38.6	34.6
3(+Ca)[b]	1	48.0	45.7	43.2	40.6	45.9	40.8	40.9	40.2	42.3	40.7
	2	45.6	44.2	39.4	36.3	39.7	38.2	42.8	40.0	36.8	32.9

[a] Mean body weight (g).
[b] Calcium carbonate (1500 ppm) added to diet.

- External examination of the newborn and weanling rats from all the litters disclosed no grossly visible abnormalities. In autopsy of the third generation weanlings, findings were negative. Histopathologic examinations of the livers and kidneys of third-generation weanling disclosed no indication of toxic effects.

2.4.1.3 NTP (1984a)

Study reference:

National Toxicology Program. 1984. Methyl salicylate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. NTP, NIEHS report NTP 84-156 (PB 84-241140)

Chapin RE and Sloane RA. Reproductive assessment by continuous breeding: Evolving study design and summaries of ninety studies. Environ Health Perspect. 1997 Feb;105 Suppl 1:199-205.

Morrissey RE, Lamb JC 4th, Morris RW, Chapin RE, Gulati DK, Heindel JJ. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fundam Appl Toxicol. 1989 Nov;13(4):747-77.

Lamb J, Gulati D, Choudhury H, Chambers R, Sabharwal. Methyl salicylate. Environmental Helath Perspectives. 1997; 105 Suppl 1.

Detailed study summary and results:

Test type

RACB (continuous breeding reproduction study) protocol: task 2 (continuous cohabitation phase) & task 4 (offspring assessment – performed because the overall response in task 2 was negative)

Test substance

- Methyl salicylate

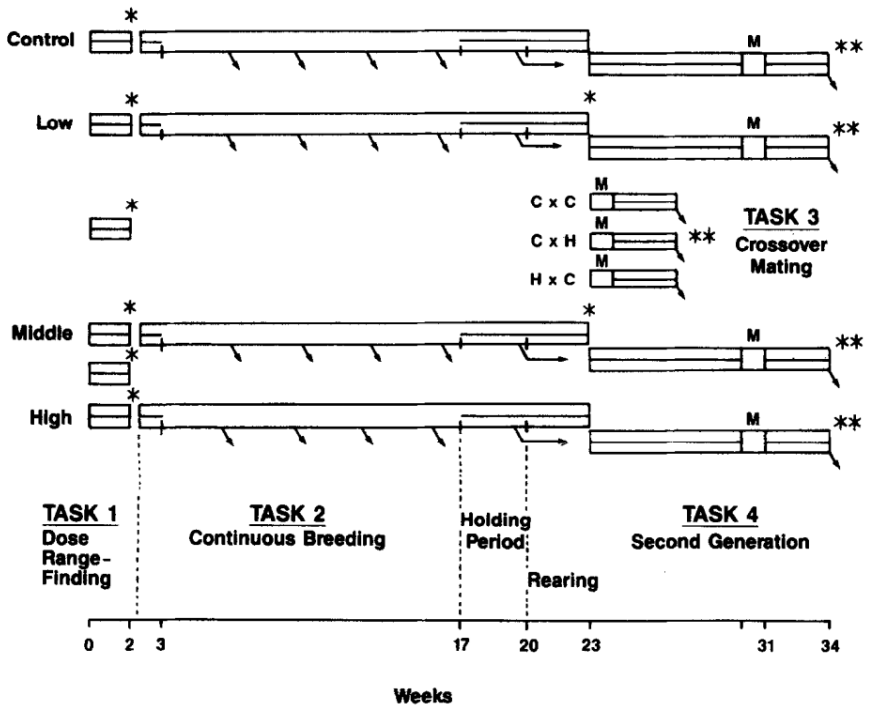
Test animals

- Swiss CD-1 mice
- n = 40/sex for control and n = 20/sex for treatment groups
- 6 weeks of age

Administration/exposure

- Oral; gavage
- 0, 25, 50, and 100 mg/kg/day based on food and water consumption, clinical signs and body weights from the Task 1 dose-range-finding study (14 days)
- Vehicle: corn oil
- Chemical was evaluated for purity and for stability in the dosing vehicle for up to 14 days. Any impurity which equalled 1.6% or more of the product was identified.

Description of test design:



- Task 2: the mice were exposed to the chemical for a 7-day premating period and were then randomly grouped as mating pairs and cohabited and treated continuously for 98 days. Data were collected on all newborns during this period (body weight, proportion of males, number of litters per pair, number of live and dead pups) within 12 hour of birth, after which each litter was discarded. After the 98-day cohabitation, the pairs were separated but continued on treatment. During the next 21 days, any final litters were delivered and kept for at least 21 days (weaning).
- Task 4: the last litter in Task 2 from the control and high dose groups was reared by the dams until weaning (postnatal day 21) and then dosed with methyl salicylate until the mating at approximately postnatal day 74. For this, male offspring were mated to female offspring from the same treatment group and the F2 litters were examined for litter size, sex and pup weight.
- Parameters assessed: see figure below.

Results and discussion

- The Cochran-Armitage test was used to test for a dose-related trend in fertility. Pairwise comparisons involving mating and fertility indices were performed using Fischer's exact test. Dose group means for number of litters, number of live pups per litter, proportion of live pups, sex ratio were tested for ordered differences using Jonckheere's test. Pairwise comparisons of treatment group means were performed by applying the Wilcoxon-Mann-Whitney U test. A Kruskal-Wallis was also performed on average pup weight. Since the number of pups in a litter may affect the average weight of the litter, an analysis of covariance was also used to test for treatment differences in average pup weight, adjusting for average litter size (live and dead pups). Pairwise comparisons wer done using a two-sided t test. For the organ weights, least-squares treatment group means were generated from an analysis of covariance (with body weight as the covariate) and were tested for overall equality using the F test and for pairwise equality using a t test. All comparisons were two-sided. The Kruskal-Wallis and Wilcoxon-Mann-Whitney U tests were also employed. Historical control data were analysed and statistical sensitivity was calculated.
- There was no adverse effect of methyl salicylate exposure on the reproductive and developmental endpoints measured.

F_0 generation Dose concentration →	25 mg/kg	50 mg/kg	100 mg/kg
General toxicity	Male, female	Male, female	Male, female
Body weight	— , —	— , —	— , —
Kidney weight[a]	•	•	•
Liver weight[a]	•	•	•
Mortality	—	—	—
Feed consumption	•	•	•
Water consumption	•	•	•
Clinical signs	—	—	—
Reproductive toxicity			
x̄ litters/pair	—	—	—
# live pups/litter; pup wt./litter	— , —	— , —	— , —
Cumulative days to litter	—	—	—
Absolute testis, epididymis weight[a]	•	•	•
Sex accessory gland weight[a] (prostate, seminal vesicle)	•	•	•
Epidid. sperm parameters (#, motility, morphology)	•	•	•
Estrous cycle length	•	•	•
Determination of affected sex (crossover)	Male	Female	Both
Dose level	•	•	•

F_1 generation Dose concentration →	•	•	100 mg/kg
General toxicity	Male, female	Male, female	Male, female
Pup growth to weaning	•	•	— , —
Mortality	•	•	— , —
Adult body weight	•	•	— , —
Kidney weight[a]	•	•	•
Liver weight[a]	•	•	— , —
Feed consumption	•	•	•
Water consumption	•	•	•
Clinical signs	•	•	— , —
Reproductive toxicity			
Fertility index	—	—	—
# live pups/litter; pup wt./litter	— , —	— , —	— , —
Absolute testis, epididymis weight[a]	•	•	— , —
Sex accessory gland weight[a] (prostate, seminal vesicle)	•	•	— , —
Epidid. sperm parameters (#, motility, morphology)	•	•	— , — , —
Estrous cycle length	•	•	•

Summary information	
Affected sex?	Unclear
Study confounders:	None
F_1 more sensitive than F_0?	No
Postnatal toxicity:	No

Legend: —, no change; •, no observation; ↑ or ↓, statistically significant change ($p<0.05$); — , —, no change in males or females. [a]Adjusted for body weight.

2.4.1.4 Anonymous (1978a)

Study reference:

Anonymous. 1978. Methyl salicylate: studies of osseous changes in the rat, reproduction in the rat and mouse and liver and kidney effects in the dog. Unpublished report to the Flavor and Extract Manufactures Association, Washington, DC. Testing laboratory: La Wall and Harrisson Research Laboratories, Inc. Report no.: 26000.

Detailed study summary and results:

Test type

2-generation study

Test substance

- Methyl salicylate

Test animals

- Wistar rats (Manor Farm)
- 25/sex/group
- Approximately 60 days old at start of the test

Administration/exposure

- 0.25 and 0.5% in Purina diet. A negative control group was maintained in the same environment as the test animals.
- Due to the volatility of methyl salicylate only enough diet for one week was prepared at any one time and this was stored in tightly closed, metal cannisters.

Description of test design:

- The animals were housed individually in plastic cages with wood chips as litter. The parent stock (F0) were maintained on their assigned diets for 60 days prior to mating. Mating was accomplished by placing a male of the same group with a female for a period of 1 week. The F0 stock were mated twice to produce F1a and F1b litters. The F1a were maintained through weaning; approximately 30 days after weaning the F0 stock were remated. Thirty males and 30 females were randomly selected from the F1b litters of each test and control group to serve as the parent stock for the F2a and F2b litters. The diets were fed to all animals, parent and young, throughout the entire test period, from initiation of the F0 stock through to the weaning of the F2b litters.
- During the littering periods the animals were observed hourly, 24 hours a day to record the number of pups per litter, their viability and condition at time of delivery. Records were maintained of total born, liveborn, number alive at 5 days and number weaned at 21 days. Litters containing more than 10 pups at day 5 were reduced to 10 pups by random selection. From these statistics stillborn, viability, lactation and reproduction indices were calculated. If a female died after weaning an initial litter the statistics of the initial litter would not be included in the overall evaluation. Records were maintained as to the total born, liveborn, live at 5 days and weaned at 21 days. Litters were reduced to 10 pups, by random selection at day 5, and day 21 statistics were suitably adjusted for this reduction. The various formulae of the indices used in evaluating the various phases of reproduction per formance were as follows:
- Stillborn = no. stillborn/total born x 100
- Viability = no. alive 5 days/no. liveborn x 100
- Lactation = no. weaned 21 days/no, alive 5 days x 100
- Reproduction no. weaned 21 days/no. liv eborn x 100

Results and discussion

- None of the young born in the litters, including the F1 and F2 litters, were observed to have any gross abnormalities. All young surviving to weaning appeared normal in respect to body growth, appearance and behaviour.
- The mating performance of the 0.25% methylsalicylate group was comparable to that of the negative control group and that of the 0.5% methyl salicylate group showed a higher number of unsuccessful matings.

TABLE VIII

RAT MATING PERFORMANCE

N* % Females (Pregnancies/Matings)

		2/2	1/2	0/2
First Generation				
Control	25	60.0	32.0	8.0
0.25%	24	70.8	25.0	4.2
0.50%	23	60.9	17.4	21.7
Second Generation				
Control	28	39.3	39.3	21.4
0.25%	30	33.3	36.7	30.0
0.50%	30	33.3	43.3	23.4
First and Second Generations				
Control	53	49.1	35.9	15.0
0.25%	54	50.0	31.5	18.5
0.50%	53	45.3	32.1	22.6

*Females mated twice

- In actual numbers the litter size for the negative control group was rather consistently larger than the litter sizes of the two levels of methyl salicylate. The 0.5% methyl salicylate group exhibited a higher number of deaths between birth and 5 days than the 0. 25% methyl salicylate and negative control groups.

TABLE IX

MEAN LITTER SIZE - BIRTH THROUGH WEANING

	Total Born	Live Born	Not Killed At Birth	Alive 5 Days	Weaned 21 Days
First Generation					
Control	11.53	11.34	11.32	11.16	9.38
0.25%	10.48	10.38	10.33	10.23	8.85
0.50%	11.03	10.63	10.53	9.38	8.09
Second Generation					
Control	9.55	8.94	8.94	8.76	8.02
0.25%	7.10	6.94	6.94	6.81	5.81
0.50%	8.49	8.33	8.33	7.58	6.41
First and Second Generations					
Control	10.61	10.22	10.21	10.04	8.75
0.25%	9.00	8.87	8.85	8.73	7.53
0.50%	9.74	9.46	9.42	8.46	7.24

- The negative control group experienced a larger number of stillborn than did either of the methyl salicylate groups; the figure ran to 6.35% for the 2 matings in the second generation. The viability indices for the 0.25% methyl salicylate group were comparable to those of the negative control group while those of the 0.5% methyl salicylate group were lower. The overall lactation indices were fairly equal between the 2 methyl salicylate groups and the negative control groups. The reproduction indices were just about equal for the 0.25% methyl salicylate group and the negative control group, both on a generation and overall basis. The 0.5% methyl salicylate had somewhat lower reproduction indices.

TABLE X

REPRODUCTION PERFORMANCE INDICES

	Stillborn	Viability	Lactation	Reproduction
First Generation				
Control	1.60	98.4	84.1	82.7
0.25%	0.95	98.6	86.8	85.5
0.50%	3.68	88.2	86.3	76.2
Second Generation				
Control	6.35	98.0	91.6	89.8
0.25%	2.27	98.1	85.3	83.7
0.50%	1.08	90.9	84.6	76.9
First and Second Generations				
Control	3.57	98.2	87.1	85.6
0.25%	1.41	98.4	86.3	84.9
0.50%	2.84	89.4	85.5	76.5

- The results of this study were analysed statistically by pooling the results of both matings in a given population. From the manner in which the various indices (stillborn, viability, lactation and reproduction) are calculated it was deemed appropriate to make an arc sine transformation so that standard analyses of variance techniques could be used to test hypotheses relative to treatment effects. Using the results of the analyses of variance, t test were run to determine the significance of treatment differences within each generation and for the pooled values of both generations.

TABLE XI

PROBABILITY (P) VALUES - TOTAL BORN/FEMALE *

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	17.52	—	28	11.25	—	53	14.21	—
0.25%	24	17.46	>.90	30	7.33	0.075	54	11.83	0.15
0.50%	23	15.35	0.40	30	4.33	0.35	53	11.94	0.15

*Twice mated females compared to controls

TABLE XII

PROBABILITY (P) VALUES - LIVE BORN/FEMALE *

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	17.24	—	28	10.54	—	53	13.70	—
0.25%	24	17.29	>0.90	30	7.17	0.10	54	11.67	0.15
0.50%	23	14.78	0.35	30	9.17	0.50	53	11.17	0.25

*Twice mated females compared to controls

TABLE XIII

PROBABILITY (P) VALUES - TOTAL WEANED/FEMALE *

	First Generation			Second Generation			Total		
	N	Mean	P	N	Mean	P	N	Mean	P
Control	25	14.26	—	28	9.44	—	53	11.72	—
0.25%	24	14.78	0.80	30	5.97	0.075	54	9.89	0.25
0.50%	23	11.26	0.20	30	7.05	0.20	53	8.87	0.075

*Twice mated females compared to controls

- Since both of the methyl salicylate groups had a lower total incidence of stillbirths over the two generations, this index was subjected to t testing. None of these three comparisons, either within each generation or for the combined generation, showed treatment differences to be significant at a level of 0.05 or less.

2.4.1.5 Anonymous (1978b)

Study reference:

Anonymous. 1978. Methyl salicylate: studies of osseous changes in the rat, reproduction in the rat and mouse and liver and kidney effects in the dog. Unpublished report to the Flavor and Extract Manufactures Association, Washington, DC. Testing laboratory: La Wall and Harrisson Research Laboratories, Inc. Report no.: 26000.

Detailed study summary and results:

Test type

2-generation study

Test substance

- Methyl salicylate

Test animals

- Mice
- 25/sex/group
- Approximately 60 days old at start of the test

Administration/exposure

- 0.25 and 0.5% in Purina diet. A negative control group was maintained in the same environment as the test animals.
- Due to the volatility of methyl salicylate only enough diet for one week was prepared at any one time and this was stored in tightly closed, metal cannisters.

Description of test design:

- The animals were housed individually in plastic cages with wood chips as litter. The parent stock (F0) were maintained on their assigned diets for 30 days prior to mating. Mating was accomplished by placing a male of the same group with a female for a period of 1 week. The F0 stock were mated twice to produce F1a and F1b litters. The F1a were maintained through weaning; approximately 30 days after weaning the F0 stock were remated. Thirty males and 30 females were randomly selected from the F1b litters of each test and control group to serve as the parent stock for the F2a and F2b litters. The diets were fed to all animals, parent and young, throughout the entire test period, from initiation of the F0 stock through to the weaning of the F2b litters.

- During the littering periods the animals were observed hourly, 24 hours a day to record the number of pups per litter, their viability and condition at time of delivery. Records were maintained of total born, liveborn, number alive at 5 days and number weaned at 21 days. Litters containing more than 10 pups at day 5 were reduced to 10 pups by random selection. From these statistics stillborn, viaility, lactation and reproduction indices were calculated.

Results and discussion

- No physical abnormalities were observed in the young of the 0.25% and 0.5% methyl salicylate groups. All young surviving to weaning exhibited normal development in respect to body growth, appearance and behaviour.
- Conception rate was higher for the two groups on methyl salicylate than for the negative control groups. The number of unsuccessful matings for the females of the negative control group was almost double that of the methyl salicylate groups.

TABLE XIV

MOUSE MATING PERFORMANCE

	N*	% Females (Pregnancies/Matings) 2/2	1/2	0/2
First Generation				
Control	20	85.0	5.0	10.0
0.25%	22	72.3	27.3	0.0
0.50%	25	72.0	28.0	0.0
Second Generation				
Control	22	9.1	72.7	18.2
0.25%	18	16.7	66.7	16.6
0.50%	16	18.9	68.8	12.4
First and Second Generation				
Control	42	45.2	40.5	14.3
0.25%	40	47.5	45.0	7.5
0.50%	41	51.2	43.9	4.9

* Females mated twice

- The negative control group although having a slightly larger litter size at birth experienced higher losses during the 21 day weaning period. At weaning the two methyl salicylate groups had equal or larger numbers of pups, on a litter basis, survive.

TABLE XV

MEAN LITTER SIZE - BIRTH THROUGH WEANING

	Total Born	Live Born	Not Killed At Birth	Alive 5 Days	Weaned 21 Days
First Generation					
Control	12.51	12.06	12.03	11.23	9.04
0.25%	10.68	10.58	10.53	10.03	9.06
0.50%	10.56	10.30	10.19	9.42	8.96
Second Generation					
Control	9.80	9.50	9.50	8.25	6.09
0.25%	11.50	11.50	11.50	11.20	7.95
0.50%	9.82	9.82	9.71	9.71	8.89
First and Second Generation					
Control	11.53	11.13	11.11	10.15	7.96
0.25%	10.95	10.88	10.84	10.41	8.70
0.50%	10.35	10.17	10.08	10.08	8.94

- The negative control group had a larger percentage of stillbirths than did the 0.25% and 0.5% methyl salicylate groups. The two salicylate groups had viability, lactation and reproduction indices that were comparable to or better than those of the negative control groups.

TABLE XVI

REPRODUCTION PERFORMANCE INDICES

	Stillborn	Viability	Lactation	Reproduction
First Generation				
Control	3.65	93.1	80.5	75.0
0.25%	0.99	94.8	90.3	85.6
0.50%	2.42	91.4	95.2	87.0
Second Generation				
Control	3.06	86.8	73.8	64.1
0.25%	0.0	97.6	70.8	69.1
0.50%	0.0	98.8	91.6	90.5
First and Second Generation				
Control	3.47	91.2	78.5	71.6
0.25%	0.65	95.7	83.6	80.0
0.50%	1.80	93.4	94.1	88.0

- The results of this mouse reproduction study did not indicate any significant treatment or generation effects attributable to the feeding of 0.25% and 0.5% methyl salicylate.

2.4.1.6 NTP (1984b)

Study reference:

National Toxicology Program. Methyl salicylate: Reproduction and fertility assessment in CD-1 mice when administered by gavage. NTP, NIEHS Report No. NTP-85-022, November 1984 (PB85-164283)

Chapin RE and Sloane RA. 1997. Reproductive assessment by continuous breeding: Evolving study design and summaries of ninety studies. Environ Health Perspect.;105 (1):199-205.

Morrissey RE, Lamb JC 4th, Morris RW, Chapin RE, Gulati DK, Heindel JJ. 1989. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fundam Appl Toxicol.;13(4):747-77.

Detailed study summary and results:

Test type

FACB (fertility assessment by continuous breeding) protocol: task 2 (continuous cohabitation phase) & task 3 (crossover mating – performed if the fertility in the task 2 is significantly affected)

GLP

Test substance

- Methyl salicylate
- Purity ≥ 99%
- Lot 703535

Test animals

- (COBS) CD-1, (ICR)BR outbred albino mice
- 40 animals/sex in the control group and 20 animals/sex in the treated groups.

Administration/exposure

- Oral, gavage
- 0, 100, 250, 500 mg/kg bw/day based on the Task 1 (14-day preliminary study). In the task 1, mice received 0, 50, 100, 250, 500 or 1000 mg/kg bw/day for 2 weeks. There was no discernible effect on body weight. Seven animals died during the study: 2 in the control group, 2 in the 50 mg/kg bw/day group and 3 at the highest dose.
- Corn oil at 10 ml/kg bw
- Dosing solutions were prepared every 2 weeks. Aliquots of various dosage formulations were sent for chemical analysis: they were within 93 and 102 % of the indicated methyl salicylate concentrations. These limits were considered acceptable.
- The actual amounts of methyl salicylate and the volume of corn oil gavaged were based on the body weight at the beginning of each week.

Description of test design:

- Task 2: Male and female mice were exposed to the chemical during 7-day premating period, after which they were randomly paired (1 male : 1 female) within each dose group. Cohabitation was continued for 100 days. Newborn litters were evaluated and immediately sacrificed. Parameters assessed: mortality, body weight, body weight gain, clinical signs, fertility index, litter per pair, live pup per litter, proportion of pups born alive, sex of pups born alive, live pup weight.
- Task 3: animals from the 500 mg/kg bw/day group were tested in a crossover mating trial to determine whether the males or females or both sexes had compromised reproductive performance when matched with control animals. Animals did not receive any treatment between days 127 (week 19) and day 155 (week 23) of the study. Parameters assessed: mating index, fertility index, live pup per litter, proportion of pups born alive, sex of pups born alive, live pup weight.

Results and discussion

TASK 2

- Eleven animals died during Task 2; 3 in the control, 2 each in the 100 and 250 mg/kg dose groups and 4 in the 500 mg/kg dose group. The cause varied from case to case but was neither chemical nor dose related. No distinct treatment related symptoms of toxicity were observed during routine health surveillance
- Methyl salicylate had no apparent effect on male or female body weights.

Treatment Group (g/kg)		Number of Animals d-0	Number of Animals d-14	Percent Mortality d(0-14)	Body Weight ± SE d-0	Body Weight ± SE d-14	Percent Change in Body Weight d(0-14)
Control	M	8	7	12.5[a]	35.6 ± 0.76	36.1 ± 0.67	+1.4
	F	8	7	12.5[a]	26.3 ± 0.66	26.7 ± 0.48	+1.5
0.05	M	8	8	0	35.2 ± 0.53	34.5 ± 0.66	-2.0
	F	8	6	25.0[a]	27.0 ± 0.48	27.1 ± 0.50	+0.4
0.10	M	8	8	0	35.4 ± 0.62	35.0 ± 0.56	-1.1
	F	8	8	0	26.7 ± 0.50	26.3 ± 0.36	-1.5
0.25	M	8	8	0	35.3 ± 0.67	34.9 ± 0.76	-1.1
	F	8	8	0	27.1 ± 0.34	25.7 ± 0.60	-5.2
0.50	M	8	8	0	34.5 ± 0.58	34.9 ± 0.72	+1.2
	F	8	8	0	27.6 ± 0.60	27.6 ± 0.65	0.0
1.00	M	8	6	25.0[a]	35.6 ± 0.52	35.1 ± 1.04	-1.4
	F	8	7	12.5[a]	26.4 ± 0.47	27.9 ± 0.65	+5.7

- The fertility index in the control and various treatment groups varied between 94 to 100%; all breeding pairs except 1 in the 100 mg/kg group delivered at least one litter. Data from breeding pairs in which one or both animals died during task 2 were excluded when computing the average number of litters pair pair, live pups per litter, proportion of pups born alive, sex ratio and live pup weight.

Table 3. Fertility of Pairs During Continuous Breeding (Task 2) Methyl Salicylate

Treatment Group	No. Fertile/ No. Cohabited	Fertility Index (%) [a,b]
Control	38/38	100
0.1 g/kg	17/18	94
0.25 g/kg	18/18	100
0.50 g/kg	16/16	100

a: $\text{Fertility Index (\%)} = \frac{\text{No. Fertile}}{\text{No. Cohabite}} \times 100$

b: Data from breeding pairs in which one partner died during cohabitation were excluded.

- There was a significant decrease ($p<0.05$) in the mean number of litters at the highest dose. The average number of pups per litter, the proportion of pups born alive, and mean live pup weight values were also significantly reduced ($p<0.05$) in the 500 mg/kg group compared to the corresponding controls.

Table 4. Reproductive Performance of Fertile Pairs During Continuous Breeding (Task 2) Methyl Salicylate

Reproductive[a] Parameter	Treatment Group (Methyl Salicylate concentration) Control	0.10 g/kg MS[c]	0.25 g/kg MS	0.5 g/kg MS
LITTERS PER PAIR	4.92 ± 0.044(38)[d]	4.82 ± 0.128(17)	4.78 ± 0.129(18)	4.50 ± 0.258(16)[e]
LIVE PUPS PER LITTER				
Male	5.73 ± 0.209(38)	5.41 ± 0.249(17)	4.75 ± 0.384(18)	4.32 ± 0.364(16)[f,g]
Female	5.55 ± 0.206(38)	5.05 ± 0.228(17)	4.83 ± 0.313(18)	3.46 ± 0.351(16)[f,h,i]
Combined	11.29 ± 0.364(38)	10.47 ± 0.348(17)	9.58 ± 0.648(18)	7.78 ± 0.635(16)[f,h]
PROPORTION OF PUPS BORN ALIVE	0.97 ± 0.010(38)	0.98 ± 0.010(17)	0.96 ± 0.017(18)	0.91 ± 0.033(16)[e,g]
SEX OF PUPS BORN ALIVE (MALES/TOTAL)	0.51 ± 0.010(38)	0.52 ± 0.016(17)	0.49 ± 0.014(18)	0.56 ± 0.024(16)[i]
LIVE PUP WEIGHT (g)				
Male	1.65 ± 0.014(38)	1.64 ± 0.020(17)	1.64 ± 0.018(18)	1.60 ± 0.023(16)
Female	1.60 ± 0.014(38)	1.59 ± 0.021(17)	1.57 ± 0.020(18)[e]	1.53 ± 0.016(16)[e,g]
Combined	1.62 ± 0.013(38)	1.62 ± 0.021(17)	1.60 ± 0.017(18)	1.57 ± 0.021(16)[e]
ADJUSTED LIVE PUP WEIGHT (g)[b]				
Male	1.67 ± 0.014(38)	1.65 ± 0.019(17)	1.63 ± 0.019(18)	1.57 ± 0.021(16)[f,h,i]
Female	1.61 ± 0.013(38)	1.59 ± 0.019(17)	1.56 ± 0.018(18)[e]	1.51 ± 0.021(16)[f,h]
Combined	1.64 ± 0.013(38)	1.62 ± 0.018(17)	1.59 ± 0.018(18)[e]	1.54 ± 0.020(16)[f,h]

a: Mean ± SE.
b: Means adjusted for total number of live and dead pups per litter by analysis of covariance.
c: MS = Methyl Salicylate.
d: Number of pairs providing data indicated in parentheses.
e: Significantly different ($p<0.05$) from the 0 g/kg group.
f: Significantly different ($p<0.01$) from the 0 g/kg group.
g: Significantly different ($p<0.05$) from the 0.1 g/kg group.
h: Significantly different ($p<0.01$) from the 0.1 g/kg group.
i: Significantly different ($p<0.05$) from the 0.25 g/kg group.

TASK 3

- First trial: both the fertility and mating index values in the control were considerably lower than expected. Only 5 out of 17 breeding pairs delivered pups in the control group. These data were considered unacceptable and Task 3 was repeated.

Table 5. Mating and Fertility of Pairs After a Mating Trial to Determine the Affected Sex in the 0.5% g/kg Dose Group (Task 1 - 1st Trial) Methyl Salicylate

Treatment Group	No. with Copulatory Plugs/ No. Cohabited	Mating Index (%)[a]	No. Fertile/ No. Cohabited	Fertility Index (%)[b]
Control Male vs. Control Female	4/17[c]	24[d]	5/17	29
0.5 g/kg Male vs. Control Female	11/18	61	13/18	72[e]
Control Male vs. 0.5 g/kg Female	8/18	44	8/18	44

a: Mating Index (%) = $\frac{\text{No. with Copulatory Plugs}}{\text{No. Cohabited}} \times 100$

b: Fertility Index (%) = $\frac{\text{No. Fertile}}{\text{No. Cohabited}} \times 100$

c: One female animal died the day after the initiation of mating; therefore, the total number of breeding pairs was reduced from 18 to 17.

d: Due to certain unknown reasons, both fertility and mating index values were considerably lower than expected. Task 3 was repeated. See data in Tables 7 and 8.

e: Significantly different ($p<0.01$) from the control male vs. control female group.

- Second trial: fertility in all three groups, including control, was poor and essentially the same. It was still not possible to determine which sex was affected by the treatment.

Table 7. Mating and Fertility of Pairs After a Mating Trial to Determine the Affected Sex in the 0.5 g/kg Dose Group (Task 3 - 2nd Trial) Methyl Salicylate

Treatment Group	No. with Copulatory Plugs /No. Cohabited	Mating Index (%)[a]	No. Fertile/ No. Cohabited	Fertility Index (%)[b]
Control Male vs. Control Female	10/17	59	7/17	41
0.5 g/kg Male vs. Control Female	7/18	39	8/18	44
Control Male vs. 0.5 g/kg Female	10/18	56	11/18	61

a: Mating Index (%) = $\frac{\text{No. with Copulatory Plugs}}{\text{No. Cohabited}} \times 100$

b: Fertility Index (%) = $\frac{\text{No. Fertile}}{\text{No. Cohabited}} \times 100$

Table 8. Reproductive Performance of Fertile Pairs to Determine the Affected Sex in the 0.5 g/kg Dose Group (Task 3 - 2nd Trial) Methyl Salicylate

Reproductive Parameter	Treatment Group: Control Male vs. Control Female	0.5 g/kg MS Male vs.[c] Control Female	Control Male vs. 0.5 g/kg MS Female
LIVE PUPS PER LITTER			
Male	2.57 ±0.75(7)[a,b]	2.63 ±0.63(8)	2.18 ±0.33(11)
Female	3.71 ±0.71(7)	2.75 ±0.77(8)	3.09 ±0.83(11)
Combined	6.29 ±1.27(7)	5.37 ±0.94(8)	5.18 ±0.89(11)
PROPORTION OF PUPS BORN ALIVE	0.87 ±0.08(7)	0.89 ±0.10(8)	0.89 ±0.06(11)
SEX OF PUPS BORN ALIVE (MALES/TOTAL)	0.42 ±0.12(7)	0.52 ±0.12(8)	0.52 ±0.12(11)
LIVE PUP WEIGHT (g)			
Male	1.81 ±0.09(6)	1.79 ±0.09(7)	1.79 ±0.03(10)
Female	1.68 ±0.05(6)	1.81 ±0.05(7)	1.69 ±0.04(10)
Combined	1.74 ±0.06(7)	1.83 ±0.07(8)	1.83 ±0.10(11)
ADJUSTED LIVE PUP WEIGHT (g)[d]			
Male	1.83 ±0.07(6)	1.80 ±0.06(7)	1.77 ±0.05(10)
Female	1.70 ±0.05(6)	1.81 ±0.05(7)	1.68 ±0.04(10)
Combined	1.76 ±0.09(7)	1.83 ±0.09(8)	1.81 ±0.08(11)

a: Mean ± SE.
b: Number of fertile pairs providing data, indicated in parenthesis.
c: MS = Methyl Salicylate.
d: Pup weight adjusted for total number of live and dead pups per litter by analysis of covariance.

2.4.1.7 FDA (2006b)

Study reference:

FDA. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029. December 13, 2006

Detailed study summary and results:

Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

Test substance

- Methyl salicylate
- Lot No. Y096
- 1001.1%

Test animals

- New Zealand White Rabbit // Kbs:NZW
- 20-22 females/group
- Age: 6 months
- Body weight on gestation day 0 ranged from 3.384 kg to 3.998 kg

Group	Test Article	Dosage Level (mg/kg/day)	Dosage Concentration (mg/mL)	Dosage Volume (mL/kg)	Number of Females
1	Methyl Salicylate	0	0	1.0	22
2	Methyl Salicylate	30	30	1.0	20
3	Methyl Salicylate	100	100	1.0	21
4	Methyl Salicylate	300	300	1.0	20

Administration/exposure

- 30, 100, 300 mg/kg/day. The high dose was based on a preliminary study for effects on embryo-fetal development (dosage: 29, 83, 250, 500 and 750 mg/kg). In this study, death was observed in the dams in the 500 and 750 mg/kg groups and an increase in the pre-implant loss index was observed in the 250 and 500 mg/kg groups. The high dose for the main study was thus set at 300 mg/kg/day, which it was expected to cause no deth of dams and to develop some toxicological signs. The middle and low doses were set at 100 and 30 mg/kg, respectively, in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepated for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within 100 ± 5%)
- The test article mixture was administered to the dorsal subcutis using a needle (22G) and syringe once daily for 13 days from day 6 to day 18 of gestation. The actual dosage volume was individually calculated from the body weight on day 6 of gestation.

Description of test design:

- Mating was conducted between males and females at the ages of 6-7 and 5-6 months, respectively. Females with full receptivity based on vulva signs were housed together with males in the male cages. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered to the dorsal subcutis using a needle (22G) and syringe once daily for 13 days from day 6 to day 18 of gestation.

- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), body weight (measured on days 0, 3, 6-19, 23, 26 and 29 of gestation), body weight gain and food consumption (measured on days 0, 3, 6-19, 23, 26 and 29 of gestation), necropsy (organs and tissues examined macroscopically; skin of the treated site, ovaries and uterus and organs and tissues with lesions were fixed in 10% neutral buffered formalin solution),
- Parameters in embryos and foetuses: number of corporea lutea, implants, early and late resorptions, dead and live foetuses were recorded, placenta was observed macroscopically, live foetuses were weighted individually and examined for external anomalies (including the oral cavity). All the live foetuses were examined macroscopically and sexed; they were sectioned into head, chest and abdomen, the brain, kidney and heart were examined for visceral anomalies; other organs were individually fixed in 10% neutral buffered formalin solution and preserved. All the carcasses of the foetuses were examined for skeletal anomalies, variations and progress of ossification (stained with Alizarin red S).
- Determination of plasma salicylic acid concentration: blood was collected 1 time at 4 hours after administration on days 6 and 18 of gestation. The concentration of salicylic acid was measured by HPLC.

Results and discussion

- As regards the body weight, body weight gain, food consumption, numbers of corporea lutea, implants, live foetuses, vertebral bodies and arches, and body weight of the live foetuses, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The indices of pre-implant loss, early and late resorption dead foetuses, total dead foetuses, placental (by type) anomalies, external (by type) anomalies, visceral (by type) variation and progress of ossification were analyzed by Wilcoxon's rank sum test. Levels of $P < 0.01$ and $P < 0.05$ were considered to be significant in all cases. The values for foetuses were recorded with each litter treated as unit.

Effects on dams:

- One dam in the 300 mg/kg group had an abortion on day 18 of gestation, and extensive vaginal haemorrhaging, blanching and pale eyes were then observed in this dam. A decrease in body weight and poor food consumption were also observed in this dam beginning on day 14 of gestation. In addition, crust of the treated site or loss of hair were observed in 1 dam in the 100 mg/kg group from day 14 to day 29 of gestation.

Summary of Clinical Signs

Rabbit №	Dose (mg/kg/day)	Day of Occurrence	Observed Clinical Sign(s)	Outcome	Necropsy Results
318	100	Gestation days 14 - 25	Crust at treatment site		
		Gestation Day 26-29	Loss of hair		
415	300	Gestation Day 18	Blanching Vaginal hemorrhage Abortion		**Uterus:** Retention of bloody material; **Vagina:** Retention of bloody material **Subcutis (treated and untreated site):** Retention of oily fluid All fetuses in the uterus had died (late resorption)

- There was no significant difference on body weight between methyl salicylate groups and the control group. A depressive trend in body weight gain (not statistically significant) was observed throughout the administration period in the 300 mg/kg group as compared with the control group.

Table 3 Body weight gains in Fo dams

Group and dose		Control			30 mg/kg			100 mg/kg			300 mg/kg		
		Body weight gain (g)											
Days of gestation	7	-4±	36	(20)	2±	31	(20)	-5±	30	(19)	-23±	55	(18)
	8	14±	46	(20)	14±	45	(20)	4±	44	(19)	-9±	80	(18)
	9	22±	53	(20)	29±	40	(20)	16±	43	(19)	-9±	72	(18)
	10	48±	63	(20)	48±	48	(20)	44±	47	(19)	16±	81	(18)
	11	55±	49	(20)	79±	54	(20)	60±	56	(19)	19±	78	(18)
	12	79±	69	(20)	90±	52	(20)	82±	55	(19)	40±	81	(18)
	13	105±	71	(20)	118±	51	(20)	115±	69	(19)	63±	87	(18)
	14	137±	92	(20)	140±	59	(20)	148±	66	(19)	110±	103	(18)
	15	157±	94	(20)	160±	82	(20)	167±	77	(19)	130±	118	(18)
	16	169±	109	(20)	198±	69	(20)	196±	74	(19)	138±	178	(18)
	17	199±	100	(20)	203±	78	(20)	218±	74	(19)	128±	214	(18)
	18	209±	95	(20)	228±	89	(20)	235±	87	(19)	185±	110	(17)
	19	221±	93	(20)	228±	93	(20)	228±	101	(19)	189±	140	(17)
	23	244±	135	(20)	247±	116	(20)	260±	133	(19)	233±	146	(17)
	26	258±	161	(20)	244±	146	(20)	268±	177	(19)	236±	180	(17)
	29	295±	193	(20)	254±	181	(20)	284±	228	(19)	248±	245	(17)

Not significantly different from control.
Values are mean±S.D. and the values in parentheses represent the number of dams.
One animal (No. 415) of the 300 mg/kg group had an abortion on day 18 of gestation.

- A significant increase in food consumption as compared with the control group was observed on day 1 of gestation in the 30 mg/kg group and on days 1-7, 9-10, 14 and 16 of gestation in the 100 mg/kg group. This effect was not dose-related.
- In necropsy of the dams on day 29 of gestation, retention of oily fluid in the subcutis of the treated site or the non-treated site (breast, axillary region or abdomen) was observed in all the dams in the control and methyl salicylate groups. Loss of hair in the treated site was observed in 1 dam in the 100 mg/kg group. In addition, light gray macule in the lung was observed in 1 dam in the 300 mg/kg group, but this change was considered to be incidental change not related to administration of methyl salicylate since it showed a low incidence. In necropsy of the dam that had an abortion, retention of oily fluid in the subcutis of the treated site and the non-treated site (axillary region) was observed. In addition, retention of bloody material in the vagina and uterus was observed, and all the foetuses in the uterus had died (late resorption).

Effects on embryo-fetal development:

- There was a significant difference in sex ratio, with a larger number of male foetuses in the 300 mg/kg group as compared with the control group (↑ 44.4%). In addition, a significant decrease in the pre-implant loss index (66.7%) as compared with the control group was observed in the 30 mg/kg group. This effect was not dose-related. The was no significant effect on the numbers of corporea lutea, implants or live foetuses, total dead foetuses, early resorption, late resorption and dead foetus indices or body weights of live foetuses. No abnormality was observed in the external examination or macroscopic observation of the placenta in the control or methyl salicylate groups.

Table 7 Observation on cesarean section of Fo dams

Group and dose	Control	30 mg/kg	100 mg/kg	300 mg/kg
No. of dams	20	20	19	17
No. of corpora lutea a)	181(9.05± 2.09)	186(9.30± 1.89)	158(8.32± 2.21)	151(8.88± 1.58)
No. of implants a)	151(7.55± 2.52)	176(8.80± 1.77)	126(6.63± 2.85)	133(7.82± 2.19)
No. of pre-implant loss b)	30(16.57)	10(5.38)*	32(20.25)	18(11.92)
No. of total dead fetuses c)	7(4.64)	18(10.23)	7(5.56)	11(8.27)
Early resorptions	4(2.65)	9(5.11)	5(3.97)	6(4.51)
Late resorptions	3(1.99)	9(5.11)	2(1.59)	5(3.76)
Dead fetuses	0	0	0	0
No. of live fetuses a)	144(7.20± 2.61)	158(7.90± 1.74)	119(6.26± 2.88)	122(7.18± 2.24)
Sex ratio of live fetuses d)	0.95(70/ 74)	1.05(81/ 77)	1.16(64/ 55)	1.71(77/ 45)*
Body weight of live fetuses (g) e)				
Male	40.85± 7.40	37.62± 5.33	40.73± 7.78	39.69± 6.52
Female	39.74± 5.90	37.89± 6.93	39.85± 8.33	38.23± 7.42
No. of live fetuses with external anomalies	0	0	0	0
No. of live fetuses with placental anomalies	0	0	0	0

*: P<0.05 (significantly different from control).
a) Values in parentheses represent mean±S.D.
b) Values in parentheses represent percentages to the number of corpora lutea.
c) Values in parentheses represent percentages to the number of implants.
d) Values in parentheses represent number of male/female fetuses.
e) Values are mean±S.D.

- Hypoplastic lung was observed in 1 fetus in the 300 mg/kg group and thymic remnant in the neck was observed in 1 fetus in the control group. There was no significant difference, however, between the incidences in the control and 300 mg/kg groups. No visceral anomalies were observed in the 30 and 100 mg/kg groups.
- Fetuses with skeletal anomalies were observed in 3 (2.08%), 8 (5.06%), 2 (1.68%) and 3 (2.46%) foetuses in the control, 30, 100 and 300 mg/kg groups respectively, but there was no significant difference between the incidences in the control and methyl salicylate groups. Considered by type of anomaly, nodulated rib was observed in 1 fetus each in the control, 100 and 300 mg/kg groups and fusion of the sternebra was observed in the 2, 8, 1 and 2 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively. There was no significant difference, however, between the control and methyl salicylate groups in terms of incidences of these anomalies by type.
- Fetuses with skeletal variation were observed in 128 (88.89%), 115 (72.78%), 100 (84.03%) and 90 (73.77%) foetuses in the control, 30, 100 and 300 mg/kg groups, respectively, but there was no significant difference between these incidences in the control and methyl salicylate groups. Considered by type of variation, cervical rib was observed in 1 fetus each in the 100 and 300 mg.kg groups; a short supernumerary rib was observed in 5, 11, 4 and 4 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively; a full supernumerary rib was observed in 124, 111, 99 and 86 fetuses in the control, 30, 100 and 300 mg/kg groups, respectively; asymmetry of the sternebra was observed in 1 fetus in the 300 mg/kg group; splitting of the sternebra was observed in 1, 3, 3 and 2 fetuses in the control, 30, 100 and 300 mg/kg grou; and incomplete ossification of lumbar or caudal centrum in 1, 3 and 1 fetuses in the control, 30 and 300 mg/kg groups, respectively. There was no significant difference between the control and methyl salicylate groups in the incidences of these variations by type. No significant difference in the progress of ossification of the vertebra, sternebra and metacarpus or phalanges was observed between the control and the methyl salicyalte groups.

Table 9 Skeletal examinations in F_1 fetuses

Group and dose	Control	30 mg/kg	100 mg/kg	300 mg/kg
No. of fetuses examined	144	158	119	122
No. of fetuses with skeletal anomalies	3(2.08)	8(5.06)	2(1.68)	3(2.46)
Nodulated rib	1(0.69)	0	1(0.84)	1(0.82)
Fusion of the sternebra	2(1.39)	8(5.06)	1(0.84)	2(1.64)
No. of fetuses with skeletal variations	128(88.89)	115(72.78)	100(84.03)	90(73.77)
Cervical rib	0	0	1(0.84)	1(0.82)
Short supernumerary rib	5(3.47)	11(6.96)	4(3.36)	4(3.28)
Full supernumerary rib	124(86.11)	111(70.25)	99(83.19)	86(70.49)
Asymmetry of the sternebra	0	0	0	1(0.82)
Splitting of the sternebra	1(0.69)	3(1.90)	3(2.52)	2(1.64)
Accessory sternebra	0	1(0.63)	0	0
Incomplete ossification of lumbar centrum	0	0	0	1(0.82)
Incomplete ossification of caudal centrum	1(0.69)	3(1.90)	0	0

Not significantly different from control.
Values in parentheses represent percentages to the number of fetuses examined.

- The plasma salicylic acid concentrations at 4 hours after administration in the 30, 100 and 300 mg/kg groups were 24.3, 62.5 and 142 µg/ml on day 6 of gestation (the first administration) and 16.5, 47.8 and 98.4 µg/ml on day 18 of gestation (the final administration), respectively.

2.4.1.8 FDA (2006c)

Study reference:

FDA. Center for Drug Evaluation and Research. 2006. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029.

Detailed study summary and results:

Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

Test substance

- Methyl salicylate
- Lot No. Y096
- 1001.1%

Test animals

- Crj:CD(SD)IGS rats
- 20 females/groups
- The body weights on day 0 of gestation were 247.5-299.2 g

Administration/exposure

- 0, 50, 100, 200 mg/kg/day. The high dose was based on a preliminary study for effects on embryo-fetal development (dosage: 75, 150, 300 and 400 mg/kg). In this study, mortality was observed in the dams in the 400 mg/kg group and a depression of body weight gain in dams, lethal on embryos, teratogenicity and suppression of fetal growth in the groups receiving 300 mg/kg or above were observed. The high dose for the main study was thus set at 200 mg/kg/day, which was expected to cause no mortality of dams and embryos and to develop some toxicological signs. The middle and low doses were set at 100 and 50 mg/kg, respectively, in a common ratio of about 2.

- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepated for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within 100 ± 5%).
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily for 12 days from day 6 to day 17 of gestation. The actual dosage volume was individually calculated from the body weight on day 6 of gestation.

Description of test design:

- Mating was started at 12 weeks of age. Nulliparous females were housed overnight with males in a 1:1 ratio. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered by subcutaneous injection once daily for 12 days from day 6 to day 17 of gestation.
- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), body weight (measured on days 0, 3 and 6-20 of gestation), body weight gain and food consumption (measured on days 1, 3 and 6-20 of gestation), necropsy (organs and tissues examined macroscopically; skin of the treated site, ovaries and uterus were fixed in 10% neutral buffered formalin solution).
- Parameters in embryos and foetuses: number of corporea lutea, implants, early and late resorptions, dead and live foetuses were recorded, placenta was observed macroscopically, live foetuses were weighted individually, sexed and examined for external anomalies (including the oral cavity). Approximately half of the foetuses from each litter were identified individually by dorsal number with an oily felt pen and fixed in Bouin's solution. The other half of the foetuses were identified individually by tattooing the four limbs after removal of the organs from the chest and abdomen and fixed in 70% ethanol. The live foetuses with external anomalies were fixed in 10% neutral buffered formalin.
- Visceral anomalies were observed in the foetuses in the control and 200 mg/kg groups that were fixed in Bouin's solution by the razor blade section method for the head and abdomen and by the microdissection method for the chest.
- The foetuses that were fixed in 70% ethanol were stained with Alizarin red S and these foetuses in all groups, including the control were examined for skeletal anomalies, variations and progress of ossification with a stereoscopic microscope.

Results and discussion

- As regards body weight, body weight gain, food consumption, numbers of corporea lutea, implants, live foetuses, vertebral bodies and arches, and body weight of the live foetuses, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The sex ratios of live foetuses were analyzed by the χ^2 test. The indices of pre-implant loss, early and late resorption, dead foetuses, total dead foetuses, placental (by type) anomalies, external (by type) anomalies, visceral (by type) anomalies, skeletal (by type) anomalies and skeletal (by type) variations and progress of ossification were analyzed by Wilcoxon's rank sum

test. Levels of P <0.01 and P<0.05 were considered to be significant in all cases. The values for foetuses were recorded with each litter treated as unit.

Effects on dams:

- No mortality occurred in the control or methyl salicylate groups. There were no abnormal signs in the control or methyl salicylate groups.
- A significant lower mean body weight as compared with the control group was observed on days 7 (3.5%), 8 (4.2%), 9 (3.4%), 10 (3.9%), 12 (3.4%) and 13 (3.6%) of gestation in the 200 mg/kg group.
- A significant decrease of body weight gain as compared with the control group was observed on day 7 of gestation in the 100 mg/kg group and during the whole treatment in the 200 mg/kg group. This decrease was ≥ 10% throughout gestation.

		Mean Body Weight Gain (g) ± SD in F_0	
Day of Treatment	**Vehicle Control**	**200 mg/kg/day**	**% Change of Control**
7	3.5 ± 2.7	-7.5 ± 4.6**	314.3%%
8	8.7 ± 5.8	-4.8 ± 6.1**	155.2%
9	12.7 ± 4.0	1.4 ± 5.3**	89%
10	19.2 ± 4.8	6.1 ± 8.3**	68%
11	23.0 ± 5.5	12.0 ± 7.6**	47.8%
12	30.3 ± 6.1	18.6± 5.7**	38.6%
13	35.2 ± 5.5	22.4 ± 8.7**	36.4%
14	40.7± 5.7	30.0± 9.1**	24.1%
15	48.4± 5.9	37.9 ± 7.1**	21.2%
16	58.2 ± 7.6	48.2 ± 8.2 **	17.2%
17	71.1 ±7.9	62.5 ± 9.2**	12.1%
18	88.0 ± 10.4	76.6 ± 10.2**	13%
19	103.7 ± 11.5	93.1 ± 11.4*	10.2%
20	120.5 ± 12.2	106.4 ± 13.1**	11.7%

* Significantly different from vehicle control (p<0.05

** Significantly different from vehicle control (p<0.01)

- A significant decrease in food consumption as compared with the control group was observed on day 7 of gestation (-11.6%) in the 100 mg/kg group and on days 6, 7 and 8 of gestation in the 200 mg/kg group (5%, 25.7% and 18.2% respectively).
- In the necropsy of the dams on day 20 of gestation, retention of oily fluid in the subcutis of the treated site was observed in all the dams in the control and methyl salicylate groups.

Effects on embryo-fetal development:

- A significant lower body weight of live foetuses as compared with the control group was observed in the 200 mg/kg group (mean body weight: -22%).
- There were no significant differences between the control and methyl salicylate groups in the numbers of corporea lutea, implants or live foetuses, pre-implant loss, total dead foetuses, early resorption, late resorption, or dead fetus indices or sex ratio.
- In the external examination, foetuses with external anomalies were observed in 1 (0.36%) and 9 (3.21%) foetuses in the control and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and 200 mg/kg groups. Considered by type of anomaly, craniorachischisis was observed in 8 fetuses from 3 litter in the 200 mg/kg group and gastroschisis was also observed in 1 fetus among them; oedema was observed in 1 fetus from another litter in the 200 mg/kg group; and vestigial tail was observed in 1 fetus from 1 litter in the control group. There was no significant difference, however, between the control and 200 mg/kg groups in terms of incidences of these anomalies by type. No abnormality was observed in the macroscopic observation of the placenta in the control or methyl salicylate groups.

Table 6 Observation on cesarean section of Fo dams

Group and dose	Vehicle control	50 mg/kg	100 mg/kg	200 mg/kg
No. of dams	20	20	20	20
No. of corpora lutea a)	306(15.30± 1.89)	319(15.95± 0.83)	304(15.20± 1.36)	305(15.25± 1.33)
No. of implants a)	296(14.80± 2.33)	304(15.20± 2.55)	289(14.45± 2.50)	300(15.00± 1.30)
No. of pre-implant loss b)	10(3.27)	15(4.70)	15(4.93)	5(1.64)
No. of total dead fetuses c)	21(7.09)	17(5.59)	10(3.46)	20(6.67)
Early resorptions	21(7.09)	17(5.59)	10(3.46)	20(6.67)
Late resorptions	0	0	0	0
Dead fetuses	0	0	0	0
No. of live fetuses a)	275(13.75± 2.22)	287(14.35± 2.58)	279(13.95± 2.54)	280(14.00± 1.81)
Sex ratio of live fetuses d)	1.15(147/128)	0.98(142/145)	1.01(140/139)	1.31(159/121)
Body weight of live fetuses (g) e)				
Male	3.64± 0.36	3.65± 0.32	3.57± 0.30	2.84± 0.32**
Female	3.45± 0.35	3.45± 0.21	3.34± 0.27	2.68± 0.21**
No. of live fetuses with external anomalies f)	1(0.36)	0	0	9(3.21)
Craniorachischisis	0	0	0	8(2.86)
Edema	0	0	0	1(0.36)
Gastroschisis	0	0	0	1(0.36)
Vestigial tail	1(0.36)	0	0	0
No. of live fetuses with placental anomalies	0	0	0	0

**: P<0.01 (significantly different from vehicle control).
a) Values in parentheses represent mean±S.D.
b) Values in parentheses represent percentages to the number of corpora lutea.
c) Values in parentheses represent percentages to the number of implants.
d) Values in parentheses represent number of male/female fetuses.
e) Values are mean±S.D.
f) Values in parentheses represent percentages to the number of live fetuses.

- Fetuses with visceral anomalies were observed in 5 (3.52%) and 11 (7.75%) foetuses in the control and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and 200 mg/kg groups. Considered by type of anomaly, ventricular septal defect was observed in 1 fetus in the 200 mg/kg group; dilatation of the ureter (unilateral) was observed in 3 and 2 fetuses in the control and 200 mg/kg groups, respectively; and thymic remnant in the neck was observed in 2 and 8 fetuses in the control and 200 mg/kg groups, respectively. There was no significant difference, however, between the control and 200 mg/kg groups in terms of incidence of these anomalies by type.
- Skeletal anomalies were observed in 3 (2.24%) and 2 (1.55%) foetuses in the 100 and 200 mg/kg groups, respectively, but there was no significant difference between the incidences in the control and methyl salicylate groups. Considered by type of anomaly, wavy ribs were observed in 3 and 1 fetuses in the 100 and 200 mg/kg groups, and fusion of the ribs was observed in 1 fetuse in the 200 mg/kg group, respectively. There was no significant difference, however, between the control and methyl salicylate groups in terms of incidences of these anomalies by type.
- Skeletal variations were observed in 14 (10.61%), 27 (19.71%), 20 (14.93%) and 97 (75.19%) fetuses in the control, 50, 100 and 200 mg/kg groups, respectively, and a significant increase in the incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of variation, cervical rib was observed in 2 and 1 fetuses in the 100 and 200 mg/kg groups; a short supernumerary rib was observed in 12 (9.09%), 23 (16.79%), 18 (13.43%) and 43 (33.33%) fetuses in the control, 50, 100 and 200 mg/kg groups, respectively; a full supernumerary rib was observed on 59 (45.74%) fetuses in the 200 mg/kg group; asymmetry of the sternebra was observed in 1 and 2 fetuses in the 50 and 200 mg/kg groups, respectively; splitting of the sternebra was observed in 1 and 3 fetuses in the 100 and 200 mg/kg groups, respectively; splitting of the thoracic vertebral body was observed in 15 (11.63%) fetuses in the 200 mg/kg group; 7 lumbar vertebra was observed in 42 (32.56%) fetuses in the 200 mg/kg group. Incomplete ossification of the thoracic centrum was observed in the 200 mg/kg group as compared with the control group. In the progress of ossification of the vertebrae, sternebra, metacarpus, metatarsus and phalanges, a significant decrease in the numbers of cervical vertebral bodies, thoracic vertebral bodies and sacrocaudal vertebral arches and bodies, and ossification indices of the 6th sternebra, metacarpus, metatarsus, proximal phalanges of the forelimb and distal phalanges of the hindlimb and a significant increase in

the numbers of the lumbar vertebral arches and bodies were observed in the 200 mg/kg group as compared with the control group.

SKELETAL EXAMINATIONS		
	Dose (mg/kg/day)	
Parameter	0	200
№ litters examined skeletally	132	129
	SKELETAL VARIATIONS Number of Subjects (percentage of the fetuses examined)	
№ of offsprings with skeletal variations (%)	14 (10.61)	97 (75.19)**
Cervical rib	0	1 (0.78)
Short supernumerary rib	12 (9.09)	43 (33.33)**
Full supernumerary rib	0	59 (45.74)**
Asymmetry of the sternebra	0	2 (1.55)
Splitting of the sternebra	0	3 (2.33)
Splitting of the thoracic vertebral body	3 (2.270	42 (32.56)**
Splitting of the lumbar vertebral body	0	15 (11.63)**
7 lumbar vertebrae	0	42 (32.56)**
In complete ossification of thoracic centrum	0	10 (7.75)*
In complete ossification of lumbar centrum	0	1 (0.78)
	PROGRESS OF OSSIFICATION Mean (± S.D.) or Number of ossification (%)[A]	
VERTRBRAE		
Cervical (body)	0.89 (0.89)	0.08 (0.15)**
Thoracic (body)	12.98 (0.070	12.69 (0.49)**
Lumbar		
- Arch (R)	6.0 (0.0.)	6.33 (0.44)**
- Arch (L)	6.0 (0.0)	6.33 (0.44)**
- Body	6 .0 (0.0)	6.33 (0.44)**
Sacrocaudal		
- Arch (R)	5.94 (0.36)	5.61 (0.47)*
- Arch (L)	5.95 (0.36)	5.60 (0.49)*
- Body	7.90 (0.72)	6.91 (0.76)**
STERNEBRAE		
- 6th	130 (98.48) A	92 (72.32) A **
METACARPUS		
- Right	513 (77.73) A	406 (62.95) A **
- Left	513 (77.73) A	394 (61.56) A**
PHALANGES OF HINDLIMBS		
- Distal	640 (98.46) A	614 (95.19) A**
METATARSUS		
- Right	540 (82.44) A	509 (79.53) A**
- Left	546 (82.73)	515 (79.84) A*

*: $p < 0.05$, significantly different from control
**: $p < 0.01$, significantly different from control
A: represent the number of ossification (ossification percentage)

2.4.1.9 FDA (2006d)

Study reference:

FDA. Center for Drug Evaluation and Research. Pharmacology / Toxicology review and evaluation. FS-67 Patch (10% Methyl salicylate & 3% l-menthol Topical patch). NDA number 22-029. December 13, 2006

Detailed study summary and results:

Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

Test substance

- Methyl salicylate
- Lot No. Y096
- 1001.1%

Test animals

- Crj:CD(SD)IGS rats
- 20 females/groups
- The body weights on day 0 of gestation were 240.8-305.1 g

Administration/exposure

- 0, 20, 60, 200 mg/kg/day. The high dose was based on a preliminary study for effects on pre- and postnatal development, including maternal function (dosage: 32, 80, 200, 300 and 500 mg/kg). In this study, mortality was observed in almost all the dams in the 500 mg/kg group. Almost none of the dams in the 300 mg/kg group delivered due to a lethal on the embryos. Furthermore, a decrease in the birth index or a decrease in the body weights of live newborns were observed in the 80 or 200 mg/kg groups, but the degree of these effects was slight. The high dose for the main study was thus set at 200 mg/kg/day, which it was expected to cause no mortality of dams and embryos and to develop some toxicological signs. The middle and low doses were set at 60 and 20 mg/kg, respectively, in a common ratio of about 3.
- Subcutaneous. Percutaneous route was planned but is difficult in a reproductive and developmental toxicity study. The subcutaneous route was chosen as a substitute route because higher plasma levels of the test article are expected with this route than with the percutaneous route.
- Dissolved in corn oil, dose volume of 1.0 mL/kg
- The stability of the test article during administration period was confirmed according to the results of analyses of the test article conducted periodically. The test article in corn oil at concentration of 5 and 500 mg/ml has been confirmed to be stable for 8 days at room temperature in a brown bottle. The test article mixture prepated for the initial and final administrations was subjected to measurement of the test article concentration and was confirmed within the predetermined concentration range (within 100 ± 5%)
- The test article mixture was administered to the dorsal subcutis using a needle (26G) and syringe once daily from day 6 of gestation to day 21 of lactation (the delivery day was designated as day 0 of lactation). The actual dosage volume was individually calculated from the body weight on day 6 of gestation during the gestation period and on day 0 of lactation during the lactation period.

Description of test design:

- Mating was started at 12 weeks of age. Nulliparous females were housed overnight with males in a 1:1 ratio. The successful copulation was confirmed by the presence of sperm in the vaginal smear. The day of successful copulation was designated as day 0 of gestation.
- The test article mixture was administered by subcutaneous injection once daily from day 6 of gestation to day 21 of lactation.
- Parameters in dams: clinical observation (twice daily during the administration period and once daily during other period), delivery and nursing conditions, including signs of delivery in the late stage of gestation, gestational days and gestation index, body weight (measured on days 0, 3, 6, 9, 12, 15, 18 and 20 of gestation and days 0, 4, 7, 10, 14, 17 and 21 of lactation), body weight gain and food consumption (measured on days 1, 3, 6, 9, 12, 15, 18 and 20 of gestation and on days 1, 4, 7, 10, 14, 17 and 21 of lactation), necropsy (organs and tissues examined macroscopically; number of implantation traces in the uterus was counted; organs and tissues with lesions, the skin of the treated site, ovaries and uterus were fixed in 10% neutral buffered formalin solution).
- Parameters in offspring:
    - state of delivery, number of litter, stillborns and live newborns were counted after delivery, birth index and stillbirth index were calculated. The live newborns were weighed, sexed and examined for external anomalies. The stillborns were examined with a floating test of the extracted lungs to determine whether thay had breathed or not.

- The litter size was standardized by random removal on postnatal day 4, such that the number of live newborns of each litter was adjusted to 8 with 4 of each sex.
- After birth and until the time of weaning (postnatal day 22), the live newborns were individually weighed on day 0, 4, 7, 14 and 21 after birth and observed for clinical signs and mortality. The viability index and the weaning index were calculated. The live newborns that died during the lactation period were fixed in pure ethanol. After weaning (postnatal day 22) and until mating, the offspring were observed for clinical signs and mortality at least once daily and their body weight and food consumption were measured once weekly. After amting, successfully copulating females were observed daily for clinical signs and their body weights were measured on days 0, 4, 7, 10 and 13 of gestation. The males and unsuccessfully copulating females were observed daily for clinical signs until necropsy.
- On the day of weaning, 3 offspring of each sex from litter containing 4 males and 4 females were necropsied, after which the organs of 1 offspring of each sex among these were weighted and moreover the other 2 offspring of each sex were submitted for skeletal examination. The remaining 1 offspring of each sex was subjected to tests of motor coordination, learning ability and emotional behaviour and to a reproductive performance test.
- All the offspring were examined for pinna detachment on postanatal day 4, piliation on postanatal day 8, incisor eruption on postnatal day 10, gait and eyelid separation on postnatal day 15, descensus testis on postnatal day 21, cleavage of balanopreputial gland on postnatal day 42 and vaginal opening on postnatal day 42. The offspring testing negative were subjected to the tests on a daily basis until they tested positive.
- All the offspring were examined for righting reflex and ipsilateral flexor reflex on postnatal day 5, visual reflex on postnatal day 16 and preyer’s reflex using audiometer on postnatal day 28. The offspring testing negative were subjected to the tests on a daily basis until they tested positive.
- On postnatal day 22, 1 offspring of each sex from a dam was sacrificed by exsanguination from the lateral iliac artery under ether anesthesia and its organs and tissues were macroscopically observed. The following organs were then isolated and weighed: heart, lungs, liver, kidneys, adrenals, brain, spleen, thymus and testes or ovaries. The organs and tissues with lesions were fixed in 10% neutral buffered formalin solution with the same organs and tissues from the control group and preserved.
- On postnatal day 22, offspring of each sex from a dam were sacrificed by exsanguination under ether anesthesia and their organs and tissues were observed macroscopically. The orgnas and tissues with lesions were be fixed in 10% neutral buffered formalin solution with the same organs and tissues from the animals from the control group and preserved. After all the carcasses were fixed in 70% ethanol and stained with alizarin red S, these in the control, 60 and 200 mg/kg groups were examined for skeletal anomalies and variations using a stereoscopic microscope.
- At the time the offspring reached 5 weeks of age, their motor coordination was examined with a rotarod by counting the number of falls during 3 minutes from the rod, which rotated 5 times a minute.
- At the time the offspring reached 6 weeks of age, their learning ability was examined with a water maze test, which was conducted for 4 consecutive days.
- At the time the offspring reached 8 weeks of age, their emotionality was examined by Hall’s method with an open field and behaviour analyse system.
- On 12-13 weeks of age, the males were paired with the females from the same group on a 1:1 basis to avoid mating within litter. The pairs were observed for successful copulation for 2 weeks. Sucessful copulation was confirmed by the presence of a vaginal plug or of sperm in a vaginal smear and the day of successful copulation was designed as day 0 of gestation.

The duration of mating required for successful copulation, copulation index and male or female fertility indices were calculated.

- The males achieving successful copulation were sacrificed and their organs and tissues were examined macroscopically. The testes and epidymis and the organs and tissues with lesions were fixed in 10% neutral buffered formalin solution. The females achieving successful copulation were sacrificed and their organs and tissues were examined macroscopically after removal of the ovaries and uterus. The organs and tissues with lesions were fixed in 10% neutral buffered formalin solution. For uncopulating females, their organs and tissues were examined macroscopically after the mating period and ovaries and uterus were fixed in 10% neutral buffered formalin solition. Dead animals after weaning were necropsied immediately after body weight measurement and their organs and tissues with lesions were fixed in 10% neutral buffered formalin solution and preserved.
- After removal of the ovaries and uterus, the unmbers of corporea lutea, implantation, dead embryos and live embryos were counted and the pre-implantation loss index and dead embryo index were calculated. The ovaries and uterus were fixed in 10% neutral buffered formalin solution.

Results and discussion

- As regards body weight, body weight gain, food consumption, gestational days, organ weights, numbers of implantation, litter, live newborns, corporea lutea and live embryos and motor coordination, learning ability and emotional behaviour test values and duration (in days) of mating required for successful copulation, the mean and standard deviations were calculated for every group, and the homogeneity of variance was tested by Bartlett's method. Comparison of the treated groups with the control group was made by Dunnett's method when the variance was found to be homogeneous or by Steel's method when the variance was not homogeneous. The gestation, copulation and male of female fertility indices and sex ratios of live foetuses were analyse by the χ^2 test. The birth, stillbirth, viability, external (by type) anomalies, skeletal (by type) anomaly, skeletal (by type) variations, pre-implantation loss and dead embryo indices and the development of differentiation and function test values were analyzed by Wilcoxon's rank sum test. Levels of $P < 0.01$ and $P < 0.05$ were considered to be significant in all cases. The values for offspring obtained before weaning were recorded with each litter treated as unit.

Effects on dams

- Two dams in the 200 mg/kg group died on day 23 of gestation. Vaginal haemorrhaging was observed in one of these dead dams and the other dam had delivered 4 live newborns and 11 stillborn upon the discovery of their death. No abnormality related to clinical signs occurred in the control or methyl salicylate groups. As regards delivery and nursing conditions, there were no changes in the control or methyl salicyalte groups. A significant prolongation of gestational days was observed in the 60 mg/kg group as compared with the control group. No significant difference in the numbers of implantation or the litter and gestation indices was observed in the methyl salicylate groups as compared with the control group.
- During the gestation period, a significantly lower mean body weight as compared with the control group was observed on days 12-20 of gestation in the 200 mg/kg group (↓ 3.7% on day 12 and ↓ 4.6% on day 20). During the lactation period, there were no significant differences in the methyl salicylate groups as compared with the vehicle control group. During the gestation period, a significant depression in body weight gain as compared with the control group was observed on days 9-20 of gestation in the 200 mg/kg group (-4.08% on day 9; -36.2% on day 12; -22.8% on day 15; -20.4% on day 18 and -15.7% on day 20). During the lactation period, no significant differences in the methyl salicylate groups showed as compared with the vehicle control group.

Table 2 Body weights in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight (g)											
Days of gestation	0	270.9±	12.0	(20)	270.6±	12.1	(20)	271.0±	10.1	(20)	270.8±	11.7	(20)
	3	292.3±	10.7	(20)	290.4±	13.1	(20)	291.1±	11.5	(20)	291.0±	10.1	(20)
	6	307.3±	10.2	(20)	303.6±	14.2	(20)	305.5±	13.0	(20)	305.0±	11.6	(20)
	9	320.3±	11.0	(20)	317.7±	16.2	(20)	320.5±	12.6	(20)	311.4±	13.6	(20)
	12	334.9±	12.9	(20)	331.2±	14.5	(20)	334.6±	14.2	(20)	322.6±	10.5*	(20)
	15	354.3±	14.6	(20)	349.8±	17.4	(20)	355.1±	15.9	(20)	341.3±	11.8*	(20)
	18	389.7±	16.7	(20)	386.7±	18.3	(20)	393.4±	20.6	(20)	370.6±	15.4**	(20)
	20	415.9±	17.0	(20)	414.4±	21.9	(20)	424.4±	23.7	(20)	396.6±	19.2*	(20)

*: P<0.05, **: P<0.01 (significantly different from vehicle control).
Values are mean±S.D. and the values in parentheses represent the number of dams.

Table 2 - continued Body weights in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight (g)											
Days of lactation	0	331.4±	17.5	(20)	324.4±	20.5	(20)	333.9±	21.1	(20)	326.5±	15.8	(18)
	4	340.8±	12.6	(20)	334.6±	16.3	(20)	346.0±	18.1	(20)	331.6±	18.1	(18)
	7	345.1±	15.3	(20)	338.1±	16.6	(20)	348.9±	18.7	(20)	337.8±	14.8	(18)
	10	345.3±	14.4	(20)	342.5±	18.2	(20)	350.6±	17.0	(20)	344.1±	17.2	(18)
	14	350.0±	15.5	(20)	338.3±	18.3	(20)	349.0±	16.2	(20)	344.6±	16.6	(18)
	17	342.0±	13.8	(20)	335.0±	15.2	(20)	342.2±	15.7	(20)	341.2±	15.4	(18)
	21	330.6±	13.1	(20)	322.5±	14.6	(20)	326.7±	16.6	(20)	331.5±	11.4	(18)

Not significantly different from vehicle control.
Values are mean±S.D. and the values in parentheses represent the number of dams.
Two dams(200 mg/kg) died on day 23 of gestation.

Table 3 Body weight gains in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight gain (g)											
Days of gestation	9	13.0±	4.2	(20)	14.1±	5.7	(20)	15.0±	6.4	(20)	6.4±	6.6**	(20)
	12	27.6±	6.1	(20)	27.6±	4.6	(20)	29.1±	7.1	(20)	17.6±	7.3**	(20)
	15	47.0±	7.2	(20)	46.2±	8.3	(20)	49.5±	8.6	(20)	36.3±	7.1**	(20)
	18	82.4±	9.4	(20)	83.1±	10.5	(20)	87.9±	13.2	(20)	65.6±	11.3**	(20)
	20	108.6±	9.6	(20)	110.8±	14.3	(20)	118.9±	16.4	(20)	91.6±	15.4**	(20)

**: P<0.01 (significantly different from vehicle control).
Values are mean±S.D. and the values in parentheses represent the number of dams.

Table 3 - continued Body weight gains in Fo dams

Group and dose		Vehicle Control			20 mg/kg			60 mg/kg			200 mg/kg		
		Body weight gain (g)											
Days of lactation	4	9.4±	14.7	(20)	10.2±	15.2	(20)	12.1±	15.8	(20)	5.0±	11.1	(18)
	7	13.7±	13.6	(20)	13.7±	17.5	(20)	15.0±	15.7	(20)	11.3±	10.5	(18)
	10	13.9±	12.7	(20)	18.1±	18.0	(20)	16.7±	17.7	(20)	17.6±	12.1	(18)
	14	18.6±	16.4	(20)	13.9±	18.0	(20)	15.1±	15.3	(20)	18.1±	15.6	(18)
	17	10.6±	16.2	(20)	10.6±	16.6	(20)	8.3±	16.2	(20)	14.7±	14.2	(18)
	21	-0.8±	13.1	(20)	-1.9±	17.8	(20)	-7.2±	17.5	(20)	5.0±	15.2	(18)

Not significantly different from vehicle control.
Values are mean±S.D. and the values in parentheses represent the number of dams.
Two dams(200 mg/kg) died on day 23 of gestation.

- During the gestation period, a significant decrease in food consumption as compared with the control group was observed on day 9 of gestation in the 200 mg/kg group (-10.2%). During the lactation period, a significant decrease in food consumption as compared with the control group was observed on days 1-7 and 14-21 of lactation in the 200 mg/kg group (-42.9%, -15.4%, -16.3%, -11.8%, -10.3% and -21.9% on days 1, 4, 7, 14, 17 and 21 respectively).
- In the necropsy of the dams on day 22 of lactation, retention of oily fluid in the subcutis of the treated site was observed in all the dams in every group. In the necropsy of the dead dams in the 200 mg/kg group, retention of oily fluid in the subcutis of the treated site was observed in all the dams and dark red macule in the stomach were observed in 1 dam among them. Furthermore, 14 dead foetuses in the uterus were observed in 1 dam and craniorachischisis was observed in 2 dead foetuses among them.

Effects on offspring

- A significant decrease in the birth index (6%) and lower body weight (9.2%) in the males were observed in live newborns in the 200 mg/kg group as compared with the control group. A trend toward a decrease in the numbers of litter and live newborns and a trend toward an increase in the stillbirth index were also observed in the 200 mg/kg group. No significant difference in the sex ratio was observed in the methyl salicylate groups as compared with the control group. No abnormality was observed in the external examination of the live newborns in any group, but craniorachischisis was observed in 4 stillborns in the 200 mg/kg group and vestigial tail and anal atresia were observed in 1 stillborn in the 60 mg/kg group.

Table 7 Terminal delivery in Fo dams and F_1 offspring

Group and dose	Vehicle Control	20 mg/kg	60 mg/kg	200 mg/kg
No. of dams	20	20	20	20 g)
Gestational days a)	21.60± 0.50	21.90± 0.55	21.95± 0.22*	21.94± 0.42
No. of implantations b)	278(13.90± 2.00)	295(14.75± 1.41)	292(14.60± 2.06)	251(13.94± 2.13)
No. of litter b)	270(13.50± 2.16)	281(14.05± 1.57)	279(13.95± 2.50)	215(11.94± 3.33)
Gestation index c)	100	100	100	90.00
No. of live newborns b)	268(13.40± 2.09)	279(13.95± 1.47)	277(13.85± 2.52)	208(11.56± 3.36)
Birth index d)	96.40	94.58	94.86	82.87*
Sex ratio of live newborns e)	1.00(134/134)	0.94(135/144)	0.95(135/142)	0.94(101/107)
Body weight of live newborns (g) a)				
Male	6.5± 0.5	6.7± 0.6	6.6± 0.4	5.9± 0.6**
Female	6.0± 0.4	6.3± 0.6	6.3± 0.5	5.6± 0.7
No. of stillborns f)				
Male	0	2	0	2
Female	2	0	2	5
Total	2(0.74)	2(0.71)	2(0.72)	7(3.26)
No. of live newborns with external anomalies	0	0	0	0

*: P<0.05, **: P<0.01 (significantly different from vehicle control).
a) Values are mean±S.D.
b) Values in parentheses represent mean±S.D.
c) Values in represent percentages to the number of pregnant animals.
d) Values in represent percentages to the number of implantations.
e) Values in parentheses represent number of male/female live newborns.
f) Values in parentheses represent percentages to the number of litters.
Craniorachischisis was observed in 4 stillborns in the 200 mg/kg group and vestigial tail and anal atresia were observed in the 1 stillborn in the 60 mg/kg group.
g) Dams with live newborns were 18 among 20 dams.

- A trend toward a decrease (92.79%) in the viability index on day 4 was observed in the 200 mg/kg group as compared with the control group (98.13%) This change was not significant and was within the range of the background data (91.32 – 99.28%) of the institution. No significant differences in the weaning index were observed in the methyl salicylate groups as compared with the control group.
- Clinical observation after weaning: an abnormality of tooth, excessive elongation of the maxillary incisors, was observed beginning on day 24 after birth in 1 female in the 200 mg/kg group and this female died after showing hypoactivity, bradypnea and lateral position on ady 31 after birth. An abnormality of tooth, excessive elongation of the maxillary incisors was also observed beginning on day 57 after birth in 2 males in the 200 mg/kg group. This change had recovered on day 87 after birth in 1 of these males but it continued until necropsy in the other male. Corectopia and dyscoria were observed beginning on day 51 after birth in 1 male and beginning on day 40 after birth in 1 female in the 200 mg/kg group, these changes continued until necropsy. Mass in the subcutis of the submacilla was additionally observed beginning on day 54 after birth in this male but this change had disappeared on day 88 after birth.
- A significant or a trend toward lower mean body weight as compared with the control group was observed throughout the lactation and maturation periods in the males and females in the 200 mg/kg group.

- A significant decrease in food consumption as compared with the control group was observed on days 28-63 after birth in the males and on days 28 and 70 after birth in the females in the 200 mg/kg group.
- A significant decrease in the differentiation indices of incisor eruption in the males and females, eyelid separation in the females and cleavage of the balanopreputial gland in the males were observed in the 200 mg/kg group as compared with the control group. No significant difference in the differentiation indices for pinna detachment, piliation, gait or descendus testis or vaginal opening was observed in the males or females in the methyl salicylate groups as compared with the control group.

Developmental Landmarks Parameter	**Day of Acquisition (for 100% of the pups)**			
Dose (mg/kg)	**0**	**20**	**60**	**200**
Males				
Balanopreputial Separation	45	47	61	72
Incisor Eruption	12	23	12	15
Females				
Eyelid Separation	15	16	15	17
Incisor Eruption	12	13	13	16

- No significant difference in the reflex indices for the righting reflex, ipsilateral flexor reflex, visual placing or preyer's reflex was observed in the males or females in the methyl salicylate groups as compared with the control group.
- Necropsy at weaning: A dilatation of the pelvic cavity was observed in 1 male in the 60 mg/kg group and 1, 2 and 1 females in the control, 20 and 60 mg/kg groups, respectively, and small testis was observed in 1 male in the 20 mg/kg group.
- Organ weight at weaning: In the males, a significant decrease in the absolute and relative weights of the liver and kidneys was observed in the 200 mg/kg group as compared with the control group. A significant decrease in the absolute weights of brain, adrenals and testes and a significant increase in the absolute weights of the brain, adrenals and testes and a significant increase in the relative weights of the brain and lungs were observed in the 200 mg/kg group as compared with the control group. In addition, a significant lower final body weight was observed in the 200 mg/kg group as compared with the control group. In the females a significant decrease in the absolute weights of the brain, heart, lungs, liver, kidneys, adrenals and ovaries and a significant increase in the relative weight of the brain were observed in the 200 mg/kg group as compared with the control group. In addition, a significant lower final body weight was observed in the 200 mg/kg group as compared with the control group.
- Skeletal examination at weaning: Skeletal anomalies were observed in 3 (3.90%), 6 (8.00%) and 20 (32.26%) offspring in the control, 60 and 200 mg/kg groups, respectively, and a significant increase in the incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of anomaly, nodulated rib was observed in 1 offspring in the 60 mg/kg group, fusion of the cervical vertebra was observed in 8 offspring (12.90%) in the 200 mg/kg group; fusion of the sternebra was observed in 3, 5 and 11 offspring in the control, 60 and 200 mg/kg groups, respectively; fusion of the thoracic vertebra was observed in 2 offspring in the 200 mg/kg group and misshapen sternebra was observed in 5 (8.06%) offspring in the 200 mg/kg group. Considering the incidences of these anomalies by type, a significant increase in the incidences of fusion of the cervical vertebra and misshapen sternebra was observed in the 200 mg/kg group as compared with the control group. Skeletal variations were observed in 20 (25.97%), 30 (40.00%) and 58 (93.55%) offspring in the control, 60 and 200 mg/kg groups, respectively, and a significant increase in their incidence was observed in the 200 mg/kg group as compared with the control group. Considered by type of variation, cervical rib was observed in 1 and 5 offspring in the control and 60 mg/kg groups,

respectively; short supernumerary rib was observed in 2 offspring in the 200 mg/kg groups; full supernumerary rib was observed in 45 (72.58%) offspring in the 200 mg/kg group; extra frontal ossification site was observed in 2 offspring in the 200 mg/kg group; accessory sternebra was observed in 11 (14.29%), 12 (16.00%) and 44 (70.97%) offspring in the control, 60 and 200 mg/kg groups, respectively; lumbarization was observed in 4 (6.45%) offspring in the 200 mg/kg group; shortened 13th rib was observed in 2 offspring in the control group; 7 lumbar vertebrae were observed in 39 (62.90%) offspring in the 200 mg/kg group; 8 lumbar vertebrae were observed in 1 (1.61%) offspring in the 200 mg/kg group; incomplete ossification of the cervical vertebra was observed in 1 (1.30%), 2 (2.67%) and 24 (38.71%) offspring in the control, 60 and 200 mg/kg groups, respectively; incomplete ossification of the thoracic vertebra was observed in 6 (7.79%), 10 (13.33%) and 43 (69.35%) offspring in the control, 60 and 200 mg/kg groups, respectively; incomplete ossification of the lumbar vertebra was observed in 22 (35.48%) offspring in the 200 mg/kg group; and incomplete ossification of the caudal vertebra was observed in 1, 4 and 3 offspring in the control, 60 and 200 mg/kg groups, respectively. Considering the incidences of these variations by type, a significant increase in the incidences of full supernumerary ribs, accessory sternebra, lumbarization, 7 lumbar vertebrae and incomplete ossification of the cervical, thoracic and lumbar centrum was observed in the 200 mg/kg group as compared with the control group.

Skeletal Examination in F_1 Offsprings			
	Dose (mg/kg/day)		
Parameter	**0**	**60**	**200**
№ litters examined skeletally	**77**	**75**	**62**
SKELETAL ANOMALIES			
№ of offsprings with skeletal anomalies (%)	3 (3.90)	6 (8.0)	20 (32.26)**
Nodulated ribs	0	1 (1.33)	0
Fusion of cervical vertebra	0	0	8 (12.90)**
Fusion of sternebra	3 (3.90)	5 (6.67)	11 (17.74)
Fusion of thoracic vertebra	0	0	2 (3.23)
Misshapen sternebra	0	0	5 (8.06)*
SKELETAL VARIATIONS			
№ of offsprings with skeletal variations (%)	20 (25.97)	30 (40)	58 (93.55)**
Cervical rib	1 (1.30)	5 (6.67)	0
Short supernumerary rib	0	0	2 (3.23)
Full supernumerary rib	0	0	45 (72.58)**
Extra frontal ossification site	0	0	2 (3.23)
Accessory sternebra	11 (14.29)	12 (16.0)	44 (70.97)**
Lumbarization	0	0	4 (6.45)*
Shortened 13th rib	2 (2.60)	0	0
7 lumbar vertebrae	0	0	39 (62.90)**
8 lumbar vertebrae	0	0	1 (1.61)
Incomplete ossification of cervical vertebrae	1 (1.30)	2 (2.67)	24 (38.71)*
Incomplete ossification of thoracic vertebrae	6 (7.79)	10 (13.33)	43 (69.35)**
Incomplete ossification of lumbar vertebrae	0	0	22 (35.48)**
Incomplete ossification of caudal vertebrae	1 (1.30)	4 (5.33)	3 (4.84)

*: $p < 0.05$, significantly different from control
**: $p < 0.15$, significantly different from control

- Motor coordination: there was no significant difference in the number of falls in the males and females between the methyl salicylate groups and the control group.
- Learning ability: A significant shortening of the second swimming time was observed in the males in the 200 mg/kg group as compared with the control group. No similar change was observed in the first or third swimming times in the same group. No significant difference in the number of errors by the males or females and swimming time in the females was observed in the methyl salicylate groups as compared with the control group.
- Emotional behaviour: A significant decrease in the number of rearing (8.1) was observed in the females in the 200 mg/kg group as compared with the control group (12.6). The value was within the

range of the background data (6.0 – 8.7) of the institution and the value for the control group was higher than the background data. No significant differences in latency or the numbers of ambulation, defecation, urination or grooming in the males and females were observed in the methyl salicylate groups as compared with the control group.

- Reproductive ability: At the first mating, copulation was not confirmed in 1, 3 and 1 pairs in the control, 20, 60 and 200 mg/kg groups, respectively and 1 and 3 females in the 60 and 200 mg/kg groups respectively were sterile after copulation. The copulation indices were accordingly 95.00, 85.00, 95.00 and 93.75 % for the control, 20, 60 and 200 mg/kg groups, respectively and the male and female fertility indices were 100, 100, 94.74 and 80.00% for the control, 20, 60 and 200 mg/kg groups respectively, results showing no significant difference between the control and methyl salicylate groups. No significant difference was observed between the numbers of days required for copulation by the control and methyl salicylate groups. When 1, 3, 1 and 1 males in the control, 20, 60 and 200 mg/kg groups, respectively, in which copulation was not observed and 2 males in the 200 mg/kg group who had no paired female were mated with non-treated females, fertility was confirmed in all cases, excluding 1 male in the 200 mg/kg group for which copulation was not observed. When 1, 3, 1 and 1 females in the control, 20, 60 and 200 mg/kg groups, respectively, in which copulation was not observed were mated with males in the same group that were confirmed to have copulated, fertility was confirmed in all cases excluding 1 female in the 60 mg/kg group in which copulation was not observed.
- Body weight in F1 dams: a significant lower mean body weight as compared with the control group was observed on day 13 of gestation in the dams in the 200 mg/kg group.
- Necropsy of offspring: In the necropsy of the males after mating, excessive elongation of the maxillary incisors was observed in 1 male in the 200 mg/kg group and corectopia and dyscoria were observed in another male in the same group. In the necropsy of the females on day 13 of gestation, corectopia and dyscoria were observed in 1 female in the 200 mg/kg group. Dilatation of the pelvic cavity was observed in 1 female each in the control and 60 mg/kg groups and gritty material in the pelvic cavity was observed in 2 and 1 females in the control and 60 mg/kg groups, respectively. In the necropsy of non-pregnant and uncopulating females, no abnormality was observed in any group. In the necropsy of the dead female in the 200 mg/kg group, a small thymus was observed. As concerns excessive elongation of the maxillary incisors observd in this female in the clinical observation, the excessive elongation site was artificially cut before death.
- Cesarean section: There were no significant differences between the control and methyl salicylate groups in the numbers of corporea lutea, implantation or live embryos or in the pre-implantation losses and dead embryo index.

2.4.1.10 Infurna *et al.* (1990)

Study reference:

Infurna R, Beyer B, Twitty L et al. 1990. Evaluation of the dermal absorption and teratogenic potential of methyl salicylate in a petroleum based grease. Teratology 41:566

Detailed study summary and results:

Test type

Equivalent or similar to OECD guideline 414; GLP not specified.

Test substance

- Methyl salicylate

Test animals

- Rats

Administration/exposure

- Dermal
- Either undiluted or in petroleum-based grease
- Treatment from gestation day 6 to 15; daily

Description of test design:

- Pregnant rats received dermal application of undiluted MeS or diluted in a petroleum based grease. Undiluted MeS was initially applied at 2000 mg/kg bw/day from GD6 but due to severe toxicity (dermal irritation and 25% mortality), the dose was reduced to 1000 mg/kg bw/day from GD10 to GD15**.**

Results and discussion

Effects on dams:

- At 2000 mg/kg bw/day: 25% mortality and severe dermal irritation

Effects on embryo-fetal development:

- At 1000 mg/kg bw/day: total resorption

2.4.1.11 Overman & White (1983)

Study reference:

Overman DO, White JA. Comparative teratogenic effects of methyl salicylate applied orally or topically to hamsters. Teratology. 1983 Dec;28(3):421-6.

Detailed study summary and results:

Test type

Study design was based on the ICH Harmonised Tripartite Guidelines related to detection of reproduction and developmental toxicities for medicinal products.

GLP compliant

Test substance

- Methyl salicylate

Test animals

- Virgin female hamster of the LVG strain

Administration/exposure

- Oral intubation at 7d9h gestation with 1750 mg/kg bw
- Topical at 7d9h gestation with 3500 or 5250 mg/kg bw

Description of test design:

- Oral administration: selected pregnant hamsters were treated by oral intubation at 7d9h gestation with a dose of 175 mg methyl salicylate per 100 g body weight. Controls received an equivalent volume of saline solution.
- Topical administration: hamster receiving topical treatment were prepared by removing the hair from the back of the neck and the back with electric clippers. Methyl salicylate was applied directly to the skin at 7d9h of gestation. To control for grooming, hamsters were anesthetized with Nembutal (13

mg) for the duration of methyl salicylate exposure. After 2 hours, the treated skin was thoroughly washed with running water. Topical controls were similarly prepared and treated with saline solultion. Nembutal controls were anesthetized and shaved but received no other treatment.

- Most embryos were recovered at 9d gestation. Some were allowed to continue their development but few survived to the age of 12d.
- Blood samples were obtained at regular intervals after treatment by heart puncture under light ether anesthesia. A 50 µl blood sample was added to 1.0 ml of modified Trinder reagent which precipitates protein and gives a purple-red color with salicylate. The samples were centrifuged and analysed spectrophotometrically reading at 546 nm against a reagent blank and a salicylate standard of known concentration. Plasma salicylate levels were calculated and plotted after oral and topical methyl salicylate treatment and the results of the 2 treatments were compared.
- To determine whether or not salicylate was actually reaching the embryos, pooled samples of older foetuses from orally treated dams were homogenized and centrifuged and the supernatant was assayed by the same procedure used with blood samples. Salicylate levels obtained from whole embryos were compared to maternal blood samples taken at the same time.

Results and discussion

Effects on embryo-fetal development:

- The defect produced by methyl salicylate treatment consisted of failure of closure of the neural tube with resulting cranium bifidum and/or spina bifida. Defects involving both the cranium and spine were more common than those involving the spine only and the failure of closure typically involved the midbrain region. No differences in the morphology of the malformation were observed between embryos receiving oral or topical treatment.

TABLE 1. Incidence of neural tube defects in 9-day hamster embryos

	Treatment					
				Methyl salicylate		
	Oral control	Topical control	Nembutal control	175 mg/100 g oral	350 mg/100 g topical	525 mg/100 g topical
Number of litters	17	6	9	35	6	19
Percent neural-tube defects	11	0	0	72	6	53

- The plasma salicylate level reached a peak of 125 mg/100 ml at about 2h after oral treatment. There followed a steady return to control levels over the newt 8-10 hours. After topical treatment, the plasma salicylate level did not reach as high a concentration as seen after oral treatment. A peak salicylate level of 50 mg/100 ml was obtained 5-6 hours after treatment and it returned to control levels over the next several hours.

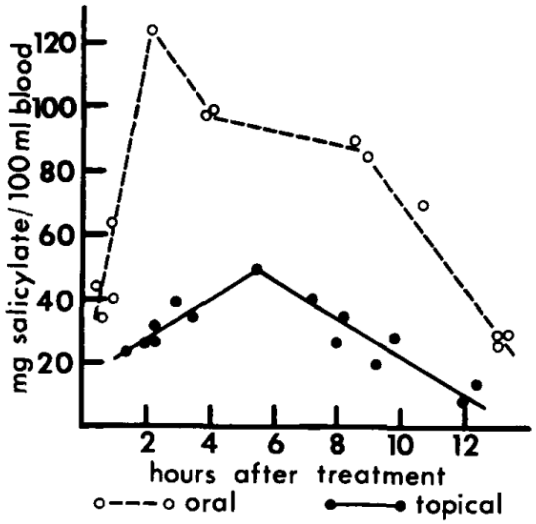


Fig. 1. Plasma salicylate levels in hamsters after oral treatment with 175 mg/100 g methyl salicylate or topical treatment with 350 mg/100 g methyl salicylate. Each point represents the average of duplicate determinations.

- Comparison of maternal and fetal salicylate levels in older foetuses showed that salicylate was reaching the fetus in some fraction of the concentration found in the mother and that the maximum level was reached more quickly in the mother than in the foetus.

TABLE 2. Comparison of maternal and fetal salicylate levels after oral methyl salicylate treatment

Time after treatment (h)	Salicylate levels (mg/100 ml fluid)	
	Maternal	Fetal
1	40.2	21.6
2	56.3	29.8
2.5	44.8	39.8

3 ENVIRONMENTAL HAZARDS

3.1 Degradation

3.1.1 Ready biodegradability (screening studies)

3.1.1.1 King (1993)

Study reference:

King JMH (1993). The biodegradability of Perfume Ingredients in the Sealed Vessel Test. Ecotoxicology Section, Unilever Research, Port Sunlight Laborator, Report 3496.

Detailed study summary and results:

Test type:

Test in accordance with the draft Ecotoxicology Section Standard Operating Procedure N° 158 01 (Operation of the Sealed Vessel Test). The sealed vessel test is a CO_2 production test based on OECD Guideline 301 B (Ready Biodegradability: CO2 Evolution Test).

Not GLP compliant

Test substance:

Methyl salicylate

Materials and methods:

The inoculum used was 10% by volume of activated non adapted sludge plant secondary effluent.

- Preparation of inoculum for exposure: The inoculum was filtered through a Whatman filter paper (541) to remove coarse particulate matter. The level of dissolved inorganic carbon (DIC) was reduced by sparging the filtered effluent with nitrogen after prior adjustment of the pH to 6.5.

TEST CONDITIONS

- Composition of medium: The mineral salts medium was as recommended in the 1988 OECD Ring Test on the harmonization of ready biodegradability tests with the following deviation: The Ferric chloride stock solution contained 0.25 g not 0.2 g. The EDTA stock solution contained 0.4g as recommended in the 1988 OECD Ring Test and not 0.5 g as specified in draft SOP 158 01.

- The sealed vessels are incubated at 20°C on a rotary shaker.

TEST SYSTEM

- Culturing apparatus: The test is conducted in 160 ml vessels (hypovials) containing 100 ml mineral salts medium inoculated with secondary effluent and the respective test or reference substance. Vessels were sealed with a butyl rubber septum and an aluminium crimp seal. The headspace in each vessel has a volume of 60 ml and, when filled with air, contains approximately 6 times the mass of oxygen required for the complete oxidation of the substance.

- Number of culture flasks/sampling time: Multiple/Five (sampling time=28 days).

- Measuring equipment: Analysis was performed using an Ionics 555 Inorganic Carbon Analyser.

- Other: The test substance was added directly to the vessels with a high quality liquid delivery syringe. The volume added (1-2 µl) is negligible compared to the total volume of 100 ml in each vessel and is not taken into consideration when determining the results.

SAMPLING

- Sampling frequency: Analysis of both the headspace and the liquid medium for CO2/DIC was performed on day numbers: 3, 8, 10, 14, 17, 21, 24, and 28.

- Initial test substance concentration: 10 mg/L based on DOC

CONTROL AND BLANK SYSTEM

- Inoculum blank: Yes

- Abiotic sterile control: No data

- Toxicity control: No data

Results:

The test substance was degraded to 68.8, 89.3 and 98.4% after 3, 8 and 28 days respectively. Even if the information is not clearly indicated in the study report, the 10-days window was met.

Table II: Percentage biodegradation of test substances

Day N°	% Biodegradation (**a**)
3	68.8
8	89.3
10	92.9
14	98.3
17	98.9
21	96.0
24	104.3
28	98.4 (**b**)
95% confid. interval	94.4 - 102.4

- The air temperature during the 28 day test period was in the range of 17-20°C. The temperature of the liquid medium in the vessels during the 28 day test period was in the range of 21.5 – 23°C.

(a) Based on nominal concentration assuming 100% purity of the named test substance.

(b) Mean of four samples only – One sample omitted after application of Dixon's test.

- The mean percentage biodegradation of methyl salicylate DQ (R548) was determined from four of the five samples only. The fifth sample was significantly lower than the mean of the remainder (2.2 % cf. 98.4%) and was omitted after application of Dixon's test. A Dixon's test value of 0.9483 was obtained which is significant at the 5% significance level (0.710).

Validity criteria (OECD):

- A test is considered valid if the difference of extremes of replicate values of the removal of the test chemical at the plateau, at the end of the test or at the end of the 10-d window, as appropriate, is less than 20% and if the percentage degradation of the reference compound has reached the pass levels by day 14. There is no raw data to check this criterion.

- If in a toxicity test, containing both the test substance and a reference compound, less than 35% degradation (based on total DOC) or less than 25% (based on total ThOD or ThCO2) occurred within 14 days, the test substance can be assumed to be inhibitory: No toxicity test is reported. However, as the substance was found to be readily biodegradable, it should not be considered as presenting a toxic effect to the inoculum tested.

- The IC content of the test substance suspension in the mineral medium at the beginning of the test must be less than 5% of the TC, and the total CO2evolution in the inoculum blank at the end of the test should not normally exceed 40 mg/l medium. If values greater than 70 mg CO2/l are obtained, the data and experimental technique should be examined critically. There is no raw data to check this criterion.

Remark of the Applicant:

Even if described as a method similar to the OECD 301B testing guideline in the test report, based on the description of the test condition, the method seems a combination of the OECD 301A and 301B testing guidelines. This should have no impact on the conclusion of the test.

3.1.1.2 Goulding et al. (1988)

Study reference:

Goulding C, Gillen C.J. and Bolton E. (1988). Biodegradation of substituted benzenes. Journal of Applied Bacteriology, 65(1), 1-5.

Detailed study summary and results:

Test type:

No guideline followed

Unsuitable test system: the inoculum used (i.e. a microbial blend consisted of five pseudomonas, one klebsiella, four rhodococci and two fungal strains) does not correspond to recommendation for ready biodegradability test. Additionnally, only primary biodegradation has been measured and not ultimate biodegradation.

Not GLP

Test substance:

Methyl salicylate

Materials and methods:

- Source of inoculum: A microbial mixture was used as inoculum. The blend consisted of five Pseudomonas, one Klebsiella, four Rhodococci and two fungal strains. The strains are held in the culture bank at InterBio Laboratories.

- Preparation of inoculum for exposure: The organisms were stabilized on a cereal base by air-drying or freeze-drying and subsequently blended. They were rehydrated in water (10g in 90 ml) shaken for 1 h at 30°C and allowed to settle for 5 min at room temperature. The supernatant fluid (10 ml) was centrifuged at 7000 rev/min for 10 min; the pellet was then washed and used to inoculate 100 ml of medium.

-Initial test substance concentration: 200 mg/L

-Duration of test: 168 h

Samples were analysed for substrate concentration (Methyl salicylate) by high performance liquid chromatography (Shimadzu LC4A system).

DETAILS ON PRETREATMENT

- Extraction: Volumes of 5 ml were extracted using 2 ml volumes of ethyl acetate in three successive extractions. The resulting organic phase was filtered through a 0.2 µm PTFE filter (Gelman) and the filtrate used directly for analysis.

IDENTIFICATION AND QUANTIFICATION OF PARENT COMPOUND

- Separation method: HPLC (Shimadzu LC4A system).

- Conditions: Samples were separated on a Nucleosil 5 C18 150 * 4.6 mm column using either acetonitrile/water or acetonitrile/acetic acid as the mobile phase and were detected using a u.v. detector (Shimadzu SPD-2AS).

TEST CONDITIONS

- Composition of medium:

KH2PO4	2.0
Na2HPO4.2H2O4	2.0
(NH4) PO4	2.0
Yeast extract	2.0
Sodium humate	0.5
Kelp extract	0.5
Tri-sodium citrate	0.1
CoSO4	0.01
ZnSO4	0.01
MnSO4.7H2O	0.2
Glucose	1.0

- pH: The pH was adjusted to 7.0.

- Agitation / Test temperature: Flasks were incubated on an orbital shaker at 150 rev/min at 30°C.

TEST SYSTEM

- Culturing apparatus: The medium was distributed in 100 ml lots in 500 ml baffled Erlenmeyer flasks. Carbon source (Methyl salicylate) was prepared as a 1000-fold stock solution in ethanol, filter sterilized (0.45 µm Millipore) and added to the basal medium after sterilization.

- Number of culture flasks/concentration: no data

SAMPLING

- Sampling frequency: Samples were removed at 24h intervals and analysed by HPLC.

CONTROL AND BLANK SYSTEM

- blank: Uninoculated control flasks were incubated in parallel.

- Reference substance: not specified

Results:

100% substrate (Methyl salicylate) removal was observed after 7 days of incubation.

Table III: Degradation rate and percentage removal of Methyl salicylate

Substrate*	Percentage removal**	Rate of removal (mg/l/h)***	Incubation time (h)
Methyl salicylate	100	4.53	168

* Initial concentration 200 mg/l

** Percentage removal is calculated by correcting for volatilization using an uninoculated control flask.

*** Rate calculated over the initial 48h period.

3.2 Acute toxicity

3.2.1 Short-term toxicity to fish

3.2.1.1 Anonymous (2000)

Study reference:

Anonymous (2000). Methyl salicylate: Acute toxicity for freshwater fish (96 Hours) *Danio rerio.* Study report 00-918013-008

Detailed study summary and results:

Test type:

OECD Guideline 203 (Fish, Acute Toxicity Test), EU Method C.1 (Acute Toxicity for Fish)

Diet: The ceasing has begun 51 hours 50 minutes before the start of the test instead of 24 hours. This deviation was not considered to have affected the quality or the interpretation of the results obatined.

Test substance:

Methyl salicylate

Materials and methods:

TEST ORGANISM

- Common name (Species): Danio rerio
- Source/Supplier: HB développement - La Fond Garel 69190 Saint Forgeux (France)
- Batch number: B991118
- Age at study initiation: Date of birth: 1999-11-18
- Length at study initiation: Mean size measured: 3.2 cm on 10 taken randomly (from 3.0 to 3.5 cm)
- Biological loading: 0.7 g/l
- Feeding during test: no

ACCLIMATION

- Acclimation period: at least 12 days
- The pH value during the acclimatization period was in the range from 7.34 to 7.79.
- The temperature during the acclimatization period was in the narrow range from 21.8 to 22.0°C.
- Acclimation conditions (same as test or not): Yes
- Type and amount of food: no data
- Feeding frequency: no data
- Feeding: The feeding was stopped 51 h 50 minutes before the test.
- Batch mortality 7 days before the test: 0%

The measured hardness of dilution water (tap water) was 120 mg of CaCO3 per litre of test medium, the measured hardness of dilution water (tap water) was 120 mg of CaCO3 per litre of test medium, the dissolved oxygen concentration was 94% minimum of the air saturation value throughout the test (air-bubbling in all test media).

TEST SYSTEM

- Test vessel: no data
- Aeration: no data
- Test type: static
- No. of organisms per vessel: 10
- No. of vessels per concentration (replicates): no data
- No. of vessels per control (replicates): no data
- Biomass loading rate: 0.7 g/l

TEST MEDIUM / WATER PARAMETERS

- Source/preparation of dilution water: Tap water

OTHER TEST CONDITIONS

- Adjustment of pH: no data
- Photoperiod: no data

EFFECT PARAMETERS MEASURED (with observation intervals if applicable): Mortality (with clinical observations)

TEST CONCENTRATIONS

- Nominal test concentrations: 0 (control), 1, 10 and 100 mg/l.

At the request of the Sponsor, the test item concentration levels were not to be checked. Then, data relating to identity, purity, both solubility and stability in water are the responsability of the Sponsor.

- Reference substance: Potassium dichromate 99.8%

Results:

Period of exposure	24 hours	48 hours	72 hours	96 hours
LC50 (mg/l)	>100	>100	>100	>100

The highest tested concentration causing a percentage of mortality ≤ 10 % was 100 mg/l at each time of exposure.

The lowest concentration causing 100% mortality was not reached in the test.

The 96h-LC50, for freshwater fish (Danio rerio) is higher than 100 mg/l.

Results with reference substance valid is considered valid

Validity criteria:

- The dissolved oxygen concentration was greater than 60% of the air saturation value throughout the test period. The minimum value obtained during the test was 94%.

- The mortality did not exceed 10% in the control group by the end of the test (0% mortality).

- The pH did not vary by more than one unit per concentration throughout the test period (maximal deviation: 0.45).

- At the request of the Sponsor, the test item concentration levels were not to be checked. No analytical monitoring has been performed although oily insoluble droplets were observed in stock solution. Therefore, it has not been demonstrated that the concentration of the substance being tested has been satisfactorily maintained through the test.

3.2.1.2 Geiger et al (1985)

Study reference:

Geiger D.L., Northcott C.E., Call D.J. and Brooke L.T. (1985). Acute toxicities of organic chemicals to fathead minnows (*Pimephales promelas*). Center for Lake Superior Environmental Studies - vol II : 203-206

Detailed study summary and results:

Test type:

Equivalent or similar to OECD Guideline 203 (Fish, Acute Toxicity Test)

Not GLP

Test substance:

ethyl salicylate / 118-61-6 / 204-265-5

PHYSICO-CHEMICAL PROPERTIES of ETHYL SALICYLATE
- Melting point: 2°C (Romanova, 1974)
- Boiling point: 234°C (FMA)
- Vapour pressure: 6.66 Pa at 20°C (calculated)
- Water solubility: 737 mg/L at 25°C (RIFM)

- Henry's law constant: no data
- log Pow: 3.09 (calculated)
- pKa: no data
- Stability in water: no data
- Stability in light: no data
- pH dependance on stability: no data

Materials and methods:

Analytical monitoring
yes
Details on sampling
All test exposure chambers were sampled at approximately mid-depth at 0 and 96h and one of each duplicate exposure chamber at 24, 48 and 72h. All samples were analysed immediatly or adequatly preserved.

Details on analytical methods
IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT
- Separation and detection method : UV-visible spectrophotometry
- Conditions (column, mobile phase, etc.):
- Detection limits (LOD, LOQ): no data
- Reproducibility in % : no data
- Linearity range: no data
- Internal or external calibration: All compound analyses included one spike and one duplicate sample for every 6 to 12 water samples. Calibration curves for each compounds were established by linear regression analysis of from 3 to 5 standards.
- Extraction recovery : results were corrected for a percent of recovery of 98.9 % (Standard Deviation : 1.2 %)

- Method of confirmation of identity of measured compound: no data

Test organisms (species)
Pimephales promelas
- Common name: Fathead minnow
- Source: From US EPA Environmental Research Laboratory- Duluth or University of WinsconsinSuperior Campus.
- Age at study initiation: 34 days
- Weight at study initiation (mean +/- SD): no data
- Length at study initiation (mean +/- SD): no data
- Loading = 0.018 g/L
- Method of breeding: Adults were held at 25 °C in flowing water with a 16h light controlled photoperiod, fed with frozen adult brine shrimp (Artemia sp.). The substrates, with intact embryos, were removed daily and placed in another 25°C bath where hatching occurred. Howevern the spawning
substrates were removed just prior to hatching at the UW-Superior culture unit, then placed in a rearing bath. Newly hatched larvea from the stock culture unit were reared in a system similar to the exposure systems at a temperature of 25°C.
- Acclimation period: no
- Feeding during test: No. Test fish were not fed 24h before or during a test.
- other: only groups of fish having healthy appearance and no history of unusual thermal exposure or abnormally high mortality rate were used in test

Test conditions:

Hardness
Mean total hardness: 44.5 mg/L as CaCO3 (SD = 0.52)
Test temperature

Grand mean of exposure temperature : 22.5 °C (pooled SD: 0.25°C)

pH

Mean arithmetic : 7.12 (SD: 0.11)

Dissolved oxygen

Grand mean of exposure dissolved oxygen concentration : 5.7 mg/L (pooled SD: 0)

Salinity

No data

Nominal and measured concentrations

Nominal concentration: not specifified

Mean measured concentrations corrected for percent recovery : 2.73, 4.82, 7.70, 14.9 and 26.2 mg/L. It should be noted that samples were not taken at 96h (the mean measured concentration was calculated from measurements at t0, 24, 48 and 72h). Twenty five fish were tested in duplicate at each control and tested concentrations.

Details on test conditions

TEST SYSTEM

A cycling proportional diluters with duplicate or quadruplicate tanks for each test concentration. The flow booster and self-siphoning flow splitting cells were removed such that the proportionally diluted stock solution fed directly into 2L battery jars which served as test chambers. Diluters were adjusted for a 0.6 dilution factor. A constant 16h photoperiod was used; while illumination, provided by a fluorescent source, produced 28-48 lumens at the surface.

TEST MEDIUM / WATER PARAMETERS

The tests conducted in Duluth used Lake Superior water which was filtered through sand and a Fram filter. Tests done on campus used dechlorinated water from the City of Superior, WI. The two waters were similar in all measured chemical parameters.

OTHER TEST CONDITIONS

- Adjustment of pH: no data
- Photoperiod and light intensity: see test system

EFFECT PARAMETERS MEASURED :

Death was the major test endpoint. The number of dead fish was noted every 24h after the beginning of the test, at which time they were also removed. Additionally, general behavioral changes were noted when they occurred.

Upon test termination, subsamples of control fish were counted into plastic freezer containers and weighed en masse. The average weight of the individual fish was calculated by dividing the number of fish into the total sample weight.

DATA ANLAYSIS:

The estimated LC50 and EC50 were calculated using the corrected averages of the analysed tank concentrations and the Trimmed Spearman-Karber Method.

Results:

At 96h, no mortality was observed at 14.9 mg/L and 100% of fishes exposed to 26.2 mg/L died. The mean measured (arith mean) LC_{50} is 19.8 mg/L.

Validity criteria:

Authors did not followed guideline and therefore did not examined validity criteria but sufficient data are available to check them.

- mortality in controls are respected (no mortality at the end of the test when no more than 10% are required)
- constant condition seemed to be maintained (temperature, pH, oxygen, flow through)
- test mean concentrations of dissolved oxygen were >60% of air saturation
- the stability of the substance cannot be estimated in regard to nominal concentration that were not mentionned in the publication. But in regard to measured concentration, it is clear that in every replicate considered separately the concentration of test substance was satisfactorily maintained during the test.

Therefore validity criteria are considered as fulfilled.

Repetition of the test :
The test has been repeated in 1983. The test conditions differed on some parameters to the test conditions described above. The principal differences were :
- analytical method : Gas Liquid Chromatography
- Grand mean of exposure temperature : 25.2 °C (pooled SD: 0.17°C)
- the test used fish that had been reared in flow-through tanks in the lab's culture unit. Larvae were fed 40 - 48h old brine shrimp nauplii in excess to times daily (one on week-end days). Embryos and larvae were cultured in water from the same source as used in the epxosures to the test chemicals.
- test system: the flow-through exposures were made with one of the following 3 systems: continuous-flow modified mini-diluter, an ABC solenoid operated electronic diluter or a single cell/glass column system
- upon test termination, individual control fish were weighed and measured. Four surviving fish each from the control, the lowest concentration and the concentration nearest the LC_{50} were preserved in 10% buffered formalin and kept for histological examination.

The results obtained were similar to the one of the first test, but with a slighlty higher LC_{50} value (i.e. 96hLC_{50} = 20.7 mg/L).

3.2.1.3 Geiger et al (1985)

Study reference:

Geiger D.L., Northcott C.E., Call D.J. and Brooke L.T. (1985). Acute toxicities of organic chemicals to fathead minnows (*Pimephales promelas*). Center for Lake Superior Environmental Studies - vol II : 141-144, 330.

Detailed study summary and results:

Test type:

Equivalent or similar to OECD Guideline 203 (Fish, Acute Toxicity Test)

Not GLP

Test substance:

PHYSICO-CHEMICAL PROPERTIES of Salicylic acid (CAS RN 69-72-7)

- Melting point: 157-160 °C
- Boiling point: 256°C
- Vapour pressure: 0.0208 Pa at 25°C
- Water solubility: 1.5 - 2.6 g/L at 20°C - 25°C
- Henry's law constant: no data
- log Pow: 0.35 - 2.64
- pKa: no data
- Stability in water: no data
- Stability in light: no data
- pH dependance on stability: no data

It should be noted that the read-across approach is performed with salicylic acid but in this study sodium salicylate was used. This data is used for the assessment of salicylic acid as a read-across approach. The

main assumption to justify the read-across approach is that the sodium ion is not significant in respect of environmental properties under consideration. Indeed, in dilute aqueous conditions of defined pH, a salt will behave no differently to the parent acid and will be fully dissociated. Hence, some properties measured in aqueous media, like aquatic ecotoxicity, for a salt can be directly read-across to the parent acid and vice versa. But suitable mass-correction have been applied.

Materials and methods:

Analytical monitoring
yes

Details on sampling
samples were taken at mid-depth at 0, 24, 48, 72, and 96h in all exposure chambers.
All samples were analysed immediatly or adequatly preserved

Details on analytical methods
IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT
- Separation method: HPLC equiped with UV-detector
- Conditions: C18 column, methanol - water mobile phase at various composition
- Calibration: external calibration established by linear regression analysis from 3 to 5 standards
- other: peak areas were used whenever possible.

Test solutions
Vehicle
no
PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: no data
- preparation of diluted stock solution: prepared in 18.9L stainless steel carbonation vessels. Lake Superior water plus the liquid toxicant were added to the vessel and stired or blended vigorously. When pH of the stock was outside the 7-8 range, pH was adjusted to 7.8 (pH of Lake Superior) with concentrated NaOH or HCl solutions
- Controls: Blank control

Test organisms (species)
Pimephales promelas
- Common name: Fathead minnow
- Source: From US EPA Environmental Research Laboratory- Duluth or University of Winscon sinSuperior Campus.
- Age at study initiation: 33 days
- Weight at study initiation (mean +/- SD): no data
- Length at study initiation (mean +/- SD): no data
- Loading = 0.032 g/L
- Method of breeding: Adults were held at 25 °C in flowing water with a 16h light controlled photoperiod, fed with frozen adult brine shrimp (Artemia sp.). Spawning was initiated with spawning substrates. The substrates with eggs and embryos were transferred daily in a rearing bath at 25 °C. La rvae were fed with 40-48h old brin shrimp nauplii. Embryos and larvae were cultured in water from the same source as used in the test.
- Acclimation period: no
- Feeding during test: No. fishes not fed 24h before test
- other: only groups of fish having healthy appearance and no history of unusual thermal exposure or abnormally high mortality rate were used in test

Test conditions
Hardness
42.7 mg/L of CaCO3 (+/- 0.5)
Test temperature

21.5°C (+/- 1.13)
pH
7.21 (+/-0.15)
Dissolved oxygen
6.0 mg/L (+/- 0.00)
test mean concentrations were >60% of saturation
Salinity
no data
Nominal and measured concentrations
TEST CONCENTRATIONS
- Spacing factor for test concentrations: no data
- nominal test concentrations: no data
- measured concentration: see table 1
Details on test conditions
TEST SYSTEM
- Test vessel: 2L battery jars which served as test chambers
- Type of flow-through: cycling proportional diluter
- No. of organisms per vessel: 20
- No. of vessels per concentration: 2
- No. of vessels per control: 2
- Biomass loading rate: 0.032 g/L

TEST MEDIUM / WATER PARAMETERS
- Source of dilution water: Lake Superior water filtered through sand and farm filter

- Alkalinity: 51.8 mg/L of CaCO3 (+/- 9.06)

- Photoperiod: 16h light / 8h dark

Results:

The LC_{50} (96h) was 1370 mg/L (CI: 1270 - 1470 mg/L), based on measured concentrations.

Lowest test substance concentration causing 100% mortality : 2275 mg/L

Table 1: measured concentration during the test

Nominal concentration (mg/L)	**Control**		**A**		**B**		**C**		**D**		**E**	
time/ replicate	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	
0h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3950	
24h	50	nd	508	nd	901	nd	1260	nd	2330	nd	4100	
48h	nd	50	nd	527	nd	880	nd	1330	nd	2280	nd	
72h	50	nd	527	nd	905	nd	1390	nd	2290	nd	3120	
96h	nd	50	nd	590	nd	864	nd	1250	nd	2120	nd	
Average	50	50	518	559	903	872	1325	1290	2310	2200	3723	
Cor av.	48	48	497	536	867	837	1272	1238	2217	2111	3573	

nd : not determined

percent recovery: 104.2 (+/- 17.9)

Method of calculating mean measured concentrations: Arithmetic mean

3.2.2 Short-term toxicity to aquatic invertebrates

3.2.2.1 Dion (1983)

Study reference:

Dion M. (1983). Détermination de la toxicité aiguë à l'égard de la daphnie de seize substances chimiques. Study report.

Detailed study summary and results:

No detailed information of this study is available.

Test type:

equivalent or similar to the Screening AFNOR Test: The duration of the test is only 24h; no analytical monitoring has been performed. Documentation is insufficient for assessment.

Not GLP

Test substance:

Methyl salicylate

Results:

Based on the results of this study (24h IC50 = 50 mg/L).

3.2.2.2 Noak (2001)

Study reference:

Noak (2001). Ethyl Salicylate: Acute Immobilisation Test (48h) to Daphnia magna STRAUS. Project-N° 001212HR / Study-N° DAI77661

Detailed study summary and results:

Test type:

OECD Guideline 202, EU Method C.2

GLP

Test substance:

ethyl salicylate / 118-61-6 / 204-265-5

Specific details on test material used for the study

PHYSICO-CHEMICAL PROPERTIES of ETHYL SALICYLATE
The following data were taken from the data gap of the Group 90 - EFFA Consortium (July 2009)
- Melting point: 2°C (Romanova, 1974)
- Boiling point: 234°C (FMA)
- Vapour pressure: 6.66 Pa at 20°C (calculated)
- Water solubility: 737 mg/L at 25°C (RIFM)
- Henry's law constant: no data
- log Pow: 3.09 (calculated)
- pKa: no data
- Stability in water: no data
- Stability in light: no data
- pH dependance on stability: no data

Materials and methods:

Details on analytical methods
- The test item was analytically verified via DOC analysis according to DIN 38409 part 3. DOC was measured in all concentration levels and control at test start (0h, fresh media).

Vehicle
No

Details on test solutions
- Stock solution: 200 mg/L were weighed out, filled up with dilution water one day prior to application and filtered before use (0.45 µm, single use).
- Dispersion treatment: 24h on a magnetic stirrer
- Test dilutions: 1:16, 1:8, 1:4, 1:2, 1:1
- Real test concentrations: 9.2, 19, 40, 84 & 165 mg/L
- Controls: Dilution water without test item tested under the same conditions as the test groups.

Test organisms
Daphnia magna
- Common name: Daphnia magna STRAUSS
- Strain: Clone 5
- Source: Institut für Wasser-, Boden- und Lufthygiene des Bundesgesundheitsamtes, Corrensplatz, D-14195 Berlin.
- Breeder: Dr.U.Noack-Laboratorium, D-31157 Sarstedt
- Age at study initiation: Less than 24h old.
- Method of breeding: Animals were bred in 2-3 L glass vessels with approximately 1.8 L culture medium. They were incubated at 21°C (temperatures of 20-25°C were tolerated) under a photoperiod of 16 h illumination (illumination strength = 1.5-5.0 µmol.m-2.s-1).
- Culture medium: Elendt M4, according to Elendt (1990), modified to a total hardness of 160 to 180 mg CaCO3/L.
- Feeding: Animals were fed ad libitum five times/week with a mix of Scenedesmus subspicatus and chlorella vulgaris, with an algae cell density of > 106 cells/mL.
ACCLIMATION
- Acclimation period: Yes, the daphnids had been acclimatized to the dilution water 2h before test started under test conditions.

Test type
Static during 48h in freshwater

Limit test
No

Test conditions:

Hardness
The total hardness of the dilution water was 260 mg/L as CaCO3.
Test temperature
The temperature of dilution water was 20.9 °C.
pH
The pH ranged from 7.35 to 7.61.
Dissolved oxygen
The dissolved oxygen concentration ranged from 8.34 to 8.68 mg/L.

Nominal and measured concentrations
Nominal concentrations of ethyl salicylate were: 12.5 , 25, 50, 100 and 200 mg/L.
Measured concentrations (via DOC analysis) were: 9.2, 19, 40, 84 and 165 mg/L.

No analytical monitoring has been performed during the test. Only initial concentrations have been measured by estimating the dissolved organic carbon

Details on test conditions

TEST SYSTEM
- Test vessel: 50 mL-glass beakers
- Volume of the study medium: 20mL
- No. of organisms per vessel: 5
- No. of vessels per concentration (replicates): 4
- No. of organisms per vessel (control): 10
- No. of vessels per control (replicates): 2

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: according to EEC 92/69 L383A C.2.
- Intervals of water quality measurement: The water parameters (dissolved oxygen concentration, pH-value) of test and control groups at the beginning of the test were measured in one replicate per concentration and control. After 48h water parameters of all replicates of the old media were measured. pH- value, oxygen concentration, temperature, conductivity and total hardness of the dilution water were measured prior to test start. DOC was measured in the new media (0h) of all test concentrations and control.

OTHER TEST CONDITIONS
- Photoperiod: 16:8 (L:D)
- Light intensity: Diffuse light, illumination range 1.5 – 5 μmol.m-2.s-1.
- Temperature: 21 ± 1°C.

EFFECT PARAMETERS MEASURED (with observation intervals if applicable) :
- In the preliminary and in the definitive test the immobilization of the daphnia was observed after 24 and 48 hours of exposure.

TEST CONCENTRATIONS
- Preliminary range finding test: It was conducted under static conditions with tree dilutions of a 100 mg/L stock solution (1:1, 1:10 & 1:100). The test solution was stirred 24h and filtered before use.
- Definitive test: It was performed with 5 dilutions of the stock dilution (200 mg/L) ranging from 1:1 to 1:16 with a dilution factor of 2 to enable the determination of 0 and 100 % immobilization after 24 and 48 hours.

- Test concentrations:

* Nominal concentrations: 12.5, 25, 50, 100 & 200 mg/L

* Measured concentrations (via DOC analysis): 9.2, 19, 40, 84 & 165 mg/L.

Reference substance (positive control)
yes - Potassiumdichromate p.a. (Merck), tested in five concentrations (0.58, 1.0, 1.8, 3.2 and 5.8 mg/L).

Results:

EC50 (24 h): 58 mg/L DOC (meas. (initial)) based on: mobility
EC50 (48 h): 28 mg/L DOC (meas. (initial)) based on: mobility

- Mortality of control: 100% survival occurred in the controls and no control sublethal effects were noted during the exposure period.

- Any observations that might cause a différence between measured and nominal values: The test item dilutions were clearly dissolved after filtration of the stock solution in all tested concentration levels throughout exposure.

Results with reference substance (positive control)
The percentage immobility for the reference item was determined after 24 h. The EC100 value was determined directly from the test results. The EC10 and EC50 with confidence interval (CI) was determined in a probability network by interpolation according to standard procedures.

EC-values after 24 h of the reference item in [mg/L]:
EC10: 1.7
EC50: 2.4 (CI 2.2 - 2.6)
EC100: 5.8
The EC50 - value of reference item potassium dichromate after 24 h is within the prescribed concen tration range of 1.0 - 2.5 mg/l of quality criteria according to AQS: DIN Guideline 38412 L 30.

Reported statistics and error estimâtes
EC100-values were deduced directly from the dose-response-relationship after 24 and 48h. There was no mathematical calculation. The concentration effect relationship is shown graphically in a probability network. EC10 and EC50-values were calculated via probit analysis. Probits according to WEBER (1986). Calculation of the confidence intervals was carried out using standard procedures according to BREITIG & TÜMPLING (1982).
Calculations were performed using software SigmaPlot rel. 2000 (2000), SPSS Corporation.

Table I: Immobilization Rates of the Preliminary Test (n=20)

	24h			48h		
Dilution*	1.Repl.	2.Repl.	MV	1.Repl.	2.Repl.	MV
1:1	40	30	35	100	100	100
1:10	0	0	0	0	0	0
1:100	0	0	0	0	0	0
Control	0	0	0	0	0	0

Repl. = replicate MV = Mean Value

* Stock solution 100 mg/L, filtrated

After 48h of exposure the total rate of immobilization of daphnia was 100% at the undiluted solution. No biologically significant effect (10%) was seen at the dilution 1:10.

Table II: Percentage of Daphnids incapable of swimming after 24 and 48h of exposure (n=20)

Immobilisation (%)										
	24h					48h				
	Replicates					Replicates				
	1.	2.	3.	4.	MV	1.	2.	3.	4.	MV
165	100	100	100	100	100	100	100	100	100	100
84	100	100	60	100	90	100	100	100	100	100
40	0	20	20	0	10	100	100	100	100	100
19	0	0	0	0	0	0	0	0	0	0
9.2	0	0	20	0	5	0	0	20	0	5
Control	0	0	0	0	0	0	0	0	0	0

MV = Mean value

The 24 and 48 h EC10 - and EC50 - values were determined by probit analysis and the 95% confidence interval for the EC50-values was calculated.

Table III: EC10-, EC50- (with confidence interval), EC100- values

	Test duration [h]	Concentration [mg/L]	Confidence interval p = 95%
EC10	24	40	-
	48	25	-
EC50	24	58	52 - 64
	48	28	27 - 29
EC100	24	165	-
	48	40	-

Validity criteria:
- In the control, including the control containing the solubilising agent, not more that 10% of the daphnids should have been immobilized: fulfilled, in the control group no Daphnia were immobilized or trapped on the surface of the water.
- The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/l in control and test vessels: fulfilled, the dissolved O_2 concentration was > 5.21 mg/L (60% of the air saturation value at the temperature used). At the end of the test the dissolved oxygen concentration ranged from 8.34 to 8.68 mg/L.
-The pH-value did not vary by more than 1 unit.
-The EC_{50}-value after 24 h of the reference item was in the range of 1.0 to 2.5 mg/L.

3.2.2.3 Kamaya (2005)

Study reference:

Kamaya Y, Fukaya Y and Suzuki K (2005). Acute toxicity of benzoic acids to the crustacean Daphnia magna, Chemosphere 59 255-261.

Detailed study summary and results:

Test type:

Equivalent or similar to OECD 202 *Daphnia* sp. Acute Immobilisation Test

Deviation : not specified

Test substance:

2-Hydroxybenzoic acid; salicylic acid / 69-72-7 / 200-712-3

The following data were taken from the REACH registration dossier of Salicylic acid (CAS RN 6972-7)
- Melting point: 157-160 °C
- Boiling point: 256°C
- Vapour pressure: 0.0208 Pa at 25°C
- Water solubility: 1.5 - 2.6 g/L at 20°C - 25°C
- Henry's law constant: no data
- log Pow: 0.35 - 2.64
- pKa: no data
- Stability in water: no data
- Stability in light: no data

pH dependance on stability: no data

Materials and methods:

- **Analytical monitoring**: yes

Details on sampling
- Concentrations: from 2 to 16 mmol/L
- Sampling method: The concentrations of the resultant solutions were checked before and after (48 h) exposure experiments.
- Sample storage conditions before analysis: no data

Details on analytical methods

DETAILS ON PRETREATMENT: no pretreatment

IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT
- Separation method (e.g. HPLC, GC): no
- Detection method: UV spectra (230, 295 nm) using a UV-visible spectrophotometer, UV mini 1240 (Shimadzu, Kyoto, Japan)
- Detection limits (LOD, LOQ) (indicate method of determination/calculation): no data

Vehicle: no

Details on test solutions

PREPARATION AND APPLICATION OF TEST SOLUTION (especially for difficult test substances)
- Method: To prepare the highest test solution, a test compound was dissolved in the aerated test medium and the pH was, where necessary, adjusted carefully with 1 mol/L NaOH solution to 7.45+ /-0.05, and then diluted to prepare a series of test solutions.
- Eluate: no data
- Differential loading: no data
- Controls: yes
- Chemical name of vehicle (organic solvent, emulsifier or dispersant): no data
- Concentration of vehicle in test medium (stock solution and final test solution): no data
- Evidence of undissolved material (e.g. precipitate, surface film, etc): no data

Test organisms (species)

Daphnia magna

- Common name: Daphnia species
- Strain: *Daphnia magna*
- Source: National Institute for Environmental Studies (NIES), Tsukuba, Japan
- Age at study initiation (mean and range, SD): <24h old (from 2-3-week-old mothers)
- Weight at study initiation (mean and range, SD): no data
- Length at study initiation (length definition, mean, range and SD): no data
- Method of breeding: Neonatal daphnids were obtained from continuous cultures in 1 liter glass beak ers at 21+/-0.3°C, in dechlorinated and conditioned tap water (total hardness =100 mg/L as CaCO3; p H 7.5+/-0.1), 16 hours light: 8 hours dark photoperiod and a density of below 20 per baker. The med ium was renewed three times a week and daphnids were fed daily with the green alga Selenastrum capricornutum NIES-35 (3.0-3.5x10^{A}8 cells/L), cultured also in our laboratory.
- Feeding during test: no data

ACCLIMATION: no data

Study design

Test type: static

Water media type: freshwater

Total exposure duration : 48h

Post exposure observation period: no data

Test conditions

Hardness: 100 mg/L CaCO3

Test temperature: 21+/-0.3°C

pH : the pH of the highest test solution was, when necessary adjusted carefully with 1 mol/L NaOH solutio n to 7.45+/-0.05 before diluting for preparing on series of test solutions.

Dissolved oxygen: no data

Nominal and measured concentrations: concentrations from 2 to 16 mmol/L (concentrations calculated from 276 to 2210 mg/L)

Details on test conditions

- Test vessel:
  - Material, size, headspace, fill volume: 50 mL glass beaker containing 40 mL of the test solution
  - Aeration: test medium before pH adjustement is aerated.
- No. of organisms per vessel: no data
- No. of vessels per concentration (replicates): 4 replicates

- No. of vessels per control (replicates): 4 replicates

TEST MEDIUM / WATER PARAMETERS
- Source/preparation of dilution water: test medium used was "moderately hard water" prepared from deionized and distilled water
- Total organic carbon: no data
- Particulate matter: no data
- Metals: no data
- Pesticides: no data
- Chlorine: no data
- Alkalinity: no data
- Ca/mg ratio: nearly 100 mg/L CaCO3
- Conductivity: no data
- Culture medium different from test medium: yes
- Intervals of water quality measurement: no data

OTHER TEST CONDITIONS
- Adjustment of pH: yes, but only for test medium and highest test solution used for lower concentr ation solutions preparation
- Photoperiod: 16-hour light to 8-hour dark
- Light intensity: no data

EFFECT PARAMETERS MEASURED:
Immobility was used as the endpoint for determining acute toxicity; the daphnids showing no movement within 15 s after gentle stirring were defined to be immobile. After 24 and 48 h, the number of immobile daphnids was recorded to determine the concentration able to achieve 50% immobilizat ion.

TEST CONCENTRATIONS
- Spacing factor for test concentrations: no data
- Justification for using less concentrations than requested by guideline: no data
- Range finding study : no data
- Reference substance (positive control): not specified

Results:

EC_{50} (48 h): 870 mg/L test mat. (nominal) based on: mobility (773-953 mg/L)
EC_{50} (24 h): 1060 mg/L test mat. (nominal) based on: mobility (898-1215 mg/L)
- Observations on body length and weight: no data
- Other biological observations: no data
- Mortality of control: no data
- Other adverse effects control: no data
- Abnormal responses: no data
- Any observations (e.g. precipitation) that might cause a difference between measured and nominal values: no data
- Effect concentrations exceeding solubility of substance in test medium: no data

Results with reference substance (positive control)

no data

Reported statistics and error estimates

EC50 values were calculated by Probit analyses. EC50 reported with 95% confidence interval.

Conclusions

2-hydroxybenzoic acid (= salicylic acid) was not harmful on *Daphnia magna*.

Justification of the read-across approach:

In this study salicylic acid has been tested. It is proposed to use this data for the assessment of the toxicity to aquatic invertebrates of methyl salicylate as a read-across approach in a weight of evidence strategy. The main assumption to justify the read-across approach is that both substances have a similar chemical structure. Both substances are 2 -hydroxybenzoic acid, but one being the methyl ester form (i.e. methyl salicylate). Therefore, both substances have the same functional groups in their chemical structure, except that methyl salicylate is the ester form of salicylic acid with an additional methyl function. This difference in the structural formula will be used in the weight of evidence approach to justify that salicylic acid is less toxic than the ester methyl form and therefore, the read-across approach with the more substituted form ethyl ester is a worst case approach.
The read-across approach is supported by the information provided in the field "Details on properties of test surrogate or analogue material". This information shows that both substances have very similar physicochemical properties (including $logK_{ow}$). But it should be noted that salicylic acid is more soluble in water than methyl salicylate (i.e. 1.5 - 2.6 g/L at 20°C - 25°C and 625 mg/L and 30°C respectively) and less volatile (i.e. 0.0208 Pa at 25°C and 13 Pa at 20°C respectively), but these differences are not expected to impact the results of the aquatic toxicity test at the concentrations tested. Even if not completely comparable due to different test conditions, the toxicity data to fish of salicylic acid is similar to methyl salicylate (i.e. $96hLC_{50}$ > 100 mg/L for both substances).
Therefore, salicylic acid is used in a read-across approach and weight of evidence strategy to assess the daphnia toxicity of methyl salicylate

3.2.3 Algal growth inhibition tests

3.2.3.1 Vryenhoef and Mullee (2010)

Study reference:

Vryenhoef H. and Mullee D.M. (2010). MethylSalicylate (CAS: 119-36-8): Algal growth inhibition test - PROJECT NUMBER: 1975/000.

Detailed study summary and results:

Test type:

According to OECD 201 and EU method C3

GLP compliant

No deviation

Test substance:

Methyl salicylate

Materials and methods:

Analytical monitoring

yes

Details on sampling

Samples were taken from the control (replicates R1 - R6 pooled) and each test group (replicates R1- R3 pooled) at 0 and 72 hours for quantitative analysis. Duplicate samples were taken at 0 and 72 hours and stored at approximately 20ºC for further analysis if necessary.

Details on analytical methods

DETAILS ON PRETREATMENT

Sample Preparation : A volume of test sample was extracted with a volume of dichloromethane to give final theoretical concentrations of 31 or 50 mg/l.

IDENTIFICATION AND QUANTIFICATION OF TEST SUBSTANCE/PRODUCT

The test item concentration in the test samples was determined by gas chromatography (GC) using an external standard. The test item gave a chromatographic profile consisting of a single peak.

- Separation method : GC

- Conditions : GC System: Agilent Technologies 5890 incorporating autosampler and workstation Column: ZB-5 (30 m x 0.53 mm id, 5 µm film), oven temperature program: initial 60ºC for 1 minute; rate: 20ºC/minute; final 200ºC for 3 minutes

Injector temperature: 250ºC

Detector temperature: 290ºC

Carrier gas and pressure: nitrogen at ~10 psi

Injection volume: 1µL

Injection mode: splitless, purge on at 1 minute

Retention time: approximately 8 minutes

- Detection method : flame ionisation detector (FID)

- Detection limits : The limit of quantitation of the analytical method was determined by calculating the sample concentration that gave a peak equivalent to ten times the baseline noise. Using this method the limit of quantitation was determined to be 0.19 mg/l.

- Linearity range: A range of standard solutions covering 0.96 to 96 mg/l (exceeding the range of the working sample concentrations) was analysed. Linearity was confirmed (R2 = 0.9994) in the range 0 to 96 mg/l.

- Internal calibration: Standard solutions of test item were prepared in dichloromethane at a nominal concentration of 50 mg/l.

- Extraction recovery : A range of preliminary test samples, accurately fortified at a known concentration of test item, was prepared and analysed. The recovery samples were prepared by addition of a standard solution of test item to a sample of test medium. A standard solution was accurately prepared by dissolving the test item in methanol. An accurate volume of the standard solution was added to a known volume of test medium to achieve the required concentration of test item. A further portion of a test sample was analysed following the addition of algal cells to assess the effects of algae on the recovery of test item from test medium.

Acceptance - 80-120% 80-120% <10%

The method is considered to be sufficiently accurate and precise for the purposes of this test. The test sample results have not been corrected for recovery.

Fortification of 6.74 mg/L plus algae: recovery of 7.73 mg/L (115%)

Fortification of 108 mg/L plus algae: recovery of 117 mg/L (108%)

Based on the results above, the presence of algal cells was found to have no significant effect on the recovery of the test item from the medium. The detection system was found to have acceptable linearity. The analytical procedure has acceptable recoveries of test item in test medium. The method of analysis has been validated and proven to be suitable for use.

Details on test solutions

Vehicle: no

PREPARATION AND APPLICATION OF TEST SOLUTION

For the purpose of the definitive test, the test item was dissolved directly in culture medium. An amount of test item (100 mg) was dissolved in culture medium and the volume adjusted to 1 litre to give a 100 mg/l stock solution from which a series of dilutions was made to give further stock solute ions of 50, 25, 12.5 and 6.25 mg/l. An aliquot (500 ml) of each of the stock solutions was separately inoculated with algal suspension (11 ml) to give the required test concentrations of 6.25, 12.5, 25, 50 and 100 mg/l. The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity. The concentration and stability of the test item in the test preparations were verified by chemical analysis at 0 and 72 hours.

Test organisms

Desmodesmus subspicatus (previous name: *Scenedesmus subspicatus*)

Details on test organisms

The test was carried out using *Desmodesmus subspicatus* strain CCAP 276/20. Liquid cultures of *Desmodesmus subspicatus* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland. Master cultures were maintained in the laboratory by the periodic replenishment of culture medium. The master cultures were maintained in the laboratory under constant aeration and constant illumination at 21 ± 1°C. Prior to the start of the test sufficient master culture was added to approximately 100 ml volumes of culture media contained in conical flasks to give an initial cell density of approximately 10E3 cells/ml. The flasks were plugged with polyurethane foam stoppers and kept under constant agitation by orbital shaker (100 – 150 rpm) and constant illumination at 24 ± 1°C until the algal cell density was approximately 10E4 - 10E5 cells/ml.

Study design

Test type: static

Water media type: freshwater

Limit test: no

Total exposure duration: 72 h

Post exposure observation period: no post exposure observation

Test conditions

Hardness: no data

Test temperature: Incubation (INFORS Multitron Version 2 incubator) at 24 ± 1ºC. Temperature was maintained at 24 ± 1ºC throughout the test.

pH: The pH values of the control cultures were observed to increase from pH 7.1 – 7.2 at 0 hours to pH 7 .6 – 7.7 at 72 hours. The pH deviation in the control cultures was less than 1.5 pH units after 72 hours and therefore was within the limits given in the Test Guidelines.

Dissolved oxygen: no data

Nominal and measured concentrations

Based on the results of the range-finding test the following test concentrations were assigned to the definitive test: 6.25, 12.5, 25, 50 and 100 mg/l. Analysis of the test preparations at 0 hours showed measured test concentrations to range from 97% to 106% of nominal. Analysis of the test preparations at 72 hours showed a concentration dependant decline in measured concentrations in the range of less than the limit of quantitation (LOQ) of the analytical method employed to 24% of nominal.

Details on test conditions

TEST SYSTEM:

As in the range-finding test 250 ml glass conical flasks were used. Six flasks each containing 100 ml of test preparation were used for the control and three flasks each containing 100 ml were used for each treatment group. The control group was maintained under identical conditions but not exposed to the test item. Pre-culture conditions gave an algal suspension in log phase growth characterised by a cell density of 1.77 x 10E5 cells per ml. Inoculation of 500 mL of test medium with 11 mL of this algal suspension gave an initial nominal cell density of 4 x 10E3 cells per mL and had no significant dilution effect on the final test concentration.

GROWTH/TEST Medium / WATERS PARAMETERS:

- Test medium: The culture medium used for both the range-finding and definitive tests was the same as that used to maintain the stock culture. The culture medium was prepared according to the OECD TG 201 (AAP-Medium) using reverse osmosis purified deionised water and the pH adjusted to 7.5 ±0.1 with 0.1N NaOH or HCl.

OTHER TEST CONDITIONS:

The flasks were plugged with polyurethane foam bungs and incubated at 24 ± 1°C under continuous illumination (intensity approximately 7000 lux) provided by warm white lighting (380 – 730 nm) and constantly shaken at approximately 150 rpm for 72 hours.

The pH of each control and test flask was determined at initiation of the test and after 72 hours exposure. The pH was measured using a WTW pH 320 pH meter. The temperature within the incubator was recorded daily.

EFFECT PARAMETERS MEASURED:

Samples were taken at 0, 25, 49 and 72 hours and the cell densities determined using a Coulter® Multisizer Particle Counter.

TEST CONCENTRATIONS:

- Spacing factor for test concentrations: 2

- Ranging finding study: YES

The test concentrations to be used in the definitive test were determined by a preliminary range-find ing test. The range-finding test was conducted by exposing *Desmodesmus subspicatus* cells to a series of nominal test concentrations of 0.10, 1.0, 10 and 100 mg/l for a period of 72 hours. The test was conducted in 250 ml glass conical flasks each containing 100 ml of test preparation and plugged with polyurethane foam bungs to reduce evaporation. Two replicate flasks were used for each control and test concentration. The test item was dissolved directly in culture medium.

An amount of test item (50 mg) was dissolved in culture medium and the volume adjusted to 500 ml to give a 100 mg/l stock solution from which a series of dilutions was made to give further stock solutions of 10, 1.0 and 0.10 mg/l. An aliquot (200 ml) of each of the stock solutions was separately inoculated with algal suspension (2 ml) to give the required test concentrations of 0.10, 1.0, 10 and 100 mg/l.

The stock solutions and each of the prepared concentrations were inverted several times to ensure adequate mixing and homogeneity. The control group was maintained under identical conditions but not exposed to the test item. At the start of the range-finding test a sample of each test and control culture was removed and the cell density determined using a Coulter® Multisizer Particle Counter. The flasks were then plugged with polyurethane foam bungs and incubated (INFORS Multitron Version 2 incubator) at 24 ± 1ºC under continuous illumination (intensity approximately 7000 lux) provided by warm white lighting (380 – 730 nm) and constantly shaken at approximately 150 rpm for 72 hours. After 72 hours the cell density of each flask was determined using a Coulter® Multisizer Particle Counter.

The results showed no significant effect on growth rate at the test concentrations of 0.10, 1.0 and 10 mg/l. However, growth was observed to be reduced at 100 mg/l.

Reference substance (positive control)

yes potassium dichromate

Any other information on materials and methods incl. tables

STABILITY PRE-TEST:

A range of preliminary test samples was prepared, analysed initially and then after storage in sealed glass vessels at ambient temperature in light and dark conditions for approximately 72 hours (equivalent to the test exposure period). In addition, a test sample was tested for stability without prior mixing (sonication) of the test sample bottle to assess for losses due to adsorption and/or insolubility. Results obtained on the stability in Aqueous Samples Using GC Analysis are described in the table below:

Table 1: Results of the stability in Aqueous Samples

Nominal concentration (mg/l)	6.25	25	100
Concentration found initially (mg/l)	7.65	28.6	113
Concentration found after storage in light conditions (mg/l)	5.43	18.8	99.6
Expressed as a percent of the initial concentration	71	66	88
Concentration found after storage in dark conditions (mg/l)	5.88	26.5	106
Expressed as a percent of the initial concentration	77	93	94
Concentration found after storage in dark conditions (mg/l) – unsonicated sample	6.15	NA	105
Expressed as a percent of the initial concentration	80	-	93

NA= Not applicable

The test item was shown to be stable in the test medium at the top level and the middle level dark sample, but showed evidence of instability in the test medium at the bottom level in all solutions and also in the light at the middle level. Information supplied by the sponsor stated that the test item reacts slowly on contact with water releasing methanol which may explain the slight decline observed. The unsonicated stability results indicated no evidence of insolubility or adherence to glass.

ADDITIONNAL STABILITY TEST

In order to confirm the exact cause for the observed decline in measured test concentrations over the 72-Hour test period a range of test samples was prepared **with and without the presence of algal cells** (4 x 103cells/ml). These samples were analysed initially and then following incubation at a temperature of 24±1°C, under continuous illumination (approximately 70000 lux) and continuous agitation at 150 rpm. The results obtained are described in the tables 2 and 3 below:

Table 2 : Stability in Aqueous Samples Incubated Under Test Conditions Determined Using GC Analysis

Nominal concentration (mg/l)	6.25	25	100
Concentration found initially (mg/l)	6.19	24.2	99.5
Expressed as a percent of the nominal concentration	99	97	100
Concentration found after storage under test conditions (mg/l)	0.494	12.4	89.9
Expressed as a percent of the nominal concentration	8	50	90

Table 3 : Stability in Aqueous Samples Prepared with the Addition of Algal Cells Incubated Under Test Conditions Determined Using GC Analysis

Nominal concentration (mg/l)	6.25	25	100
Concentration found initially (mg/l)	5.90	23.9	92.6
Expressed as a percent of the nominal concentration	94	96	93
Concentration found after storage under test conditions (mg/l)	<LOQ	<LOQ	77.2
Expressed as a percent of the nominal concentration	-	-	77

LOQ = Limit of quantitation

The test item was shown to be unstable under test conditions at the lowest and middle concentrations, a 10% decline in measured concentration was observed at the highest test concentration. A significant decline in measured concentration was observed at the lowest and middle concentrations in the presence of algal cells indicating that the test item adsorbed to the alga cells present. As such it was considered that the decline in measured test concentrations observed during the definitive test was due to a combination of both the unstable nature of the test item and also adsorption of the test item to the algal cells present.

Evaluation of data

- Comparison of growth rates

The average specific growth rate for a specified period is calculated as the logarithmic increase in biomass. The average specific growth rate over the test duration was calculated for each replicate control and test item vessel using the nominally inoculated cell concentration as the starting value rather than the measured starting value in order to increase the precision of the calculation. In addition the section by section specific growth rate (days 0-1, 1-2 and 2-3) was calculated for the control cultures and the results examined in order to determine whether the growth rate remained constant.Percentage inhibition of growth rate for each replicate test item vessel was calculated.

- Comparison of Yield

Yield is calculated as the increase in biomass over the exposure period. For each test concentration and control the mean value for yield along with the standard deviation was calculated.

- Determination of ECx values
For each individual test vessel (mean values for yield), percentage inhibition (arithmetic axis) was plotted against test concentration (logarithmic axis) and a line fitted by computerised interpolation using the Xlfit software package (IDBS). ECxvalues were then determined from the equation for the fitted line. Where appropriate 95% confidence limits for the EC50values were calculated, using the simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

- Statistical analysis
One way analysis of variance incorporating Bartlett's test for homogeneity of variance (Sokal and Rohlf 1981) and Dunnett's multiple comparison procedure for comparing several treatments with a control (Dunnett 1955) was carried out on the growth rate and yield data after 72 hours for the control and all test concentrations to determine any statistically significant differences between the test and control groups. All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

Results:

- The results obtained with **nominal concentrations** of methyl salicylate were as follows:

72h-ErC50 = 27 mg/L (95% CL : 24 - 31 mg/L) (growth rate)
72h-EbC50 = 13 mg/L (95% CL: 12 – 14 mg/L) (biomass)
72h-LOEC = 12.5 mg/L (growth rate and biomass)
72h-NOEC = 6.25 mg/L (growth rate and biomass)

- The results obtained with the **geometric mean of the measured concentrations** of methyl salicylate were as follows:

72h-ErC50 = 1.6 mg/L (95% CL: 1.5 - 1.7 mg/L) (growth rate)
72h-EbC50 = 1.1 mg/L (95% CL: 1.1 - 1.2 mg/L) (biomass)
72h-NOEC = 1.1 mg/L (growth rate and biomass)
72h-NOEC = 0.79 mg/L (growth rate and biomass)

- A positive control used potassium dichromate as the reference item. The reference item was tested at concentrations of 0.0625, 0.125, 0.25, 0.50 and 1.0 mg/l.

ErC50 (0 – 72 h) : 0.49 mg/l
EyC50 (0 – 72 h) : 0.18 mg/l, 95% confidence limits 0.16 – 0.21 mg/l
NOEC based on growth rate: 0.0625 mg/l
NOEC based on yield: 0.0625 mg/l
LOEC based on growth rate: 0.125 mg/l
LOEC based on yield: 0.125 mg/l
The results from the positive control with potassium dichromate were within the normal ranges for this reference item.

Reported statistics and error estimates

There were no statistically significant differences between the control and 6.25 mg/l test concentration (P>=0.05), however all other test concentrations were significantly different (P<0.05) and, therefore the "No Observed Effect Concentration" (NOEC) based on growth rate was 6.25 mg/l. Correspondingly the "Lowest Observed Effect Concentration" (LOEC) based on growth rate was 12.5 mg/l.

Statistical analysis of the yield data was carried out as for growth rate. There were no statistically significant differences between the control and 6.25 mg/l test concentration (P>=0.05), however all other test concentrations were significantly different (P<0.05) and, therefore the "No Observed Effect Concentration"

(NOEC) based on yield was 6.25 mg/l. Correspondingly the "Lowest Observed Effect Concentration" (LOEC) based on yield was 12.5 mg/l.

Details on results submitted by the registrant (with the FR-MSCA's caution)

- Exponential growth in the control : yes

- Observation of abnormalities : All test and control cultures were inspected microscopically at 72 hours. After 72 hours there were no abnormalities detected in the control or test cultures at 6.25, 12.5 and 25 mg/l, however few intact cells were observed to be present in the test cultures at 50 and 100 mg/l.

- Any stimulation of growth found in any treatment: no

- Any observations (e.g. precipitation) that might cause a difference between measured and nominal values: at the start of the test all control and test cultures were observed to be clear colourless solutions. After the 72-Hour test period all control, 6.25, 12.5 and 25 mg/l test cultures were observed to be pale green dispersions whilst the 50 and 100 mg/l test cultures were observed to be clear colourless solutions.

- Effect concentrations exceeding solubility of substance in test medium: no

- Verification of test concentrations:

Analysis of the test preparations at 0 hours showed measured test concentrations to range from 97% to 106% of nominal. **Analysis of the test preparations at 72 hours showed a concentration dependant decline in measured concentrations in the range of less than the limit of quantitation (LOQ) of the analytical method employed to 24% of nominal.** This decline was inline with the preliminary stability analyses conducted which indicated slight instability over the test period. The further decline in measured test concentrations was considered by the author to be due to adsorption of the test item to the algal cells present. Additional stability analyses conducted under identical test conditions confirmed the unstable nature of the test item over the 72-Hour exposure period and the losses of the test item when the algal cells are present.

Overall the decline in measured concentrations followed a concentration dependent pattern with greater losses being observed at the lower test concentrations. This effect was considered to be due to there being greater numbers of algal cells in the lower concentrations and hence greater surface area for adsorption to occur. Whilst the recovery analyses performed in the presence of algal cells showed that under the conditions employed for these samples no significant adsorption to algal cells occurred, these results do not preclude long term adsorption during the test in the presence of algal cells undergoing logarithmic growth. Adsorption was not a factor in the stability analyses conducted as no algal cells were present.

According to current regulatory advice that in cases where a decline in measured concentrations is observed, geometric mean measured concentrations should be used for calculating EC_{50} values, results were not only based on nominal concentrations but also on the geometric mean measured test concentrations in order to give a "worst case" analysis of the data. In cases where the measured concentration was less than the LOQ of the analytical method following current regulatory advice a value of half the LOQ (i.e. 0.095 mg/l) was used to enable calculation of the geometric mean measured concentration.

The high level of methyl salicylate decrease observed in this study when algae are present in the assay medium has been attributed by the author, to adsorption of the substance on algal cells. **This was only an unverified hypothesis that is contradicted by the substance water solubility and log Kow that do not let predict such a strong adsorption**. The moderate volatility of methyl salicylate has been taken into account in the experiment by using flasks plugged with polyurethane foam bungs. This leads to investigate whether methyl salicylate metabolization could take place in algae.

At first, an absorption rather than an adsorption of the substance could be expected, as suggested by results from Wang and Lay (1989): the ionizable salicylic acid showed unexpectedly high bioconcentration (log BCF = 3) in green algae, which may be related to its effect on algal growth. Methyl salicylate could then be expected to accumulate similarly in algae. However, absorption is sometimes not necessary when the enzymes are released onto the medium, as shown in results hereafter.

Algae are known to have various enzymes able to metabolize a variety of chemicals (Tagaki, 2010):

- oxidases (P450 enzymes in marine algae (Pflugmacher and Sandermann 1998b))
- reductases (most relevant information on reductive metabolism mechanism and the enzymes involved is limited to algae and aquatic macrophytes),
- glucose transferases (salicylic acid has been shown to be metabolized by *Lemna gibba* by glucosidation (Ben-Tal and Cleland (1982)),
- sulfotransferases (Enzyme activity has been reported in various aquatic organisms such as bivalva, crustacea, green algae),
- glutathione-S-transferases (In emergent and submergent aquatic macrophytes and various algae, GST activity toward typical substrates were detected in both microsomal and cytosolic factions (Pflugmacher and Steinberg 1997, Pflugmacher et al. 1999, 2000),
- acyltransferases (Acetylation and formylation reactions are also known to occur mostly in algae, macrophytes, and bivalves).
- esterases, among them carboxyesterases (for example, Baeza-Squiban et al. (1988, 1990) have shown that the green alga Dunaliella sp. can hydrolyze deltamethrin to the corresponding acid and alcohol by carboxyesterase released into medium. Propanil was hydrolyzed by various green and bluegreen algae to 3,4-dichloroaniline (Wright and Maule 1982)).

Moreover, esterase activity is used as a basis for algal bioassays, as shown for example in Regel *et al.* (2002), a study that investigated the potential for using algal esterase activity of *Microcystis aeruginosa* and *Selenastrum capricornutum* as a rapid measure of biological effects of some pollutants, or a more recent report published on the Royal Society of Chemistry website (Shi and Tai, 2010) using algal esterase as indicator for environmental toxicity. This shows that esterase activity is a very common feature in algae.

In the light of results above, the methyl salicylate decay observed in 72h in presence of algae could be explained by (absorption and) metabolization of the substance rather than to adsorption that is not supported by physico-chemical properties. In such a situation, the nominal EC_{50} and NOEC found reflects the combination of a toxic effect of methyl salicylate, and its hydrolysis catalyzed by the algal carboxyesterases, giving methanol and salicylic acid that are not harmful for algal growth (ECHA, 2010), (Henschel *et al*., 1997). When a substance hydrolyses fast (at least within the tests time span), it is relevant to assess the hydrolysis products hazard for assessing the substance itself.

In these conditions, **nominal concentrations more appropriately reflect overall methyl salicylate toxicity (substance itself and hydrolysis products released within the test time span)**, rather than the concentrations measured after 72h contact with algae that reflects substance residues remaining in the assay solution. **Therefore a chronic toxicity classification is not appropriate, as reflected by the NOEC expressed as nominal concentration: 6.25 mg/L.**

Conclusions

Based on the results of this study (**72h ErC_{50} = 27 mg/L, NOEC = 6.25 mg/L**), methyl salicylate is considered as harmful in short-term conditions and not having long lasting effets in the aquatic organisms tested in accordance with CLP Regulation.

FR-MSCA comments

The rapporteur member state disagrees with the registrant's conclusions. Since the important decline observed in measured concentrations, the results obtained with the geometric mean of the measured concentrations rather than the nominal concentrations had to be considered in the calculation of the EC_{50} and NOEC values. Consequently, the relevant EC_{50} is 1.6 mg/L and the relevant NOEC is 0.79 mg/L, and methyl salicylate need to be classified as chronic hazard category 3, H412 with respect to the aquatic environment according to the Regulation (EC) No 1272/2008.

Validity criteria (According to OECD 201, 2006):

- The following data show that the cell concentration of the control cultures increased by a factor of 59 after 72 hours. This increase was in line with the OECD Guideline that states the enhancement must be at least by a factor of 16 after 72 hours.
Mean cell density of control at 0 hours : 4.64 x 10^3cells per ml
Mean cell density of control at 72 hours : 2.72 x 10^5cells per ml
- The mean coefficient of variation for section by section specific growth rate for the control cultures was 22% and hence satisfied the validation criterion given in the OECD Guideline which states the mean must not exceed 35%.
- The coefficient of variation for average specific growth rate for the control cultures over the test period (0 – 72 h) was 3% and hence satisfied the validation criterion given in the OECD Guideline which states that this must not exceed 7%.

Table 1: Cell Densities and pH Values in the Definitive Test

Nominal concentration (mg/L)		pH	Cell density (cells* per mL)				pH
		0h	0h	25h	49h	72h	72h
Control	R1	7.2	4.18E+03	1.11E+04	6.37E+04	3.08E+05	7.7
	R2	7.2	4.72E+03	1.50E+04	6.38E+04	3.02E+05	7.7
	R3	7.2	4.09E+03	1.29E+04	4.27E+04	2.02E+05	7.7
	R4	7.2	5.09E+03	1.22E+04	4.74E+04	2.77E+05	7.7
	R5	7.2	4.71E+03	1.25E+04	5.12E+04	2.88E+05	7.6
	R6	7.1	5.02E+03	1.31E+04	6.75E+04	2.56E+05	7.6
	Mean		4.64E+03	1.28E+04	5.60E+04	2.72E+05	
6.25	R1	7.1	5.31E+03	1.47E+04	1.45E+04	3.35E+05	7.6
	R2	7.1	5.06E+03	1.11E+04	1.33E+04	3.14E+05	7.6
	R3	7.1	5.07E+03	1.18E+04	2.34E+04	3.12E+05	7.6
	Mean		5.15E+03	1.25E+04	1.71E+04	3.21E+05	
12.5	R1	7.1	6.67E+03	1.13E+04	2.25E+04	1.75E+05	7.6
	R2	7.1	5.41E+03	1.23E+04	1.92E+04	1.31E+05	7.6
	R3	7.1	5.27E+03	1.14E+04	1.89E+04	1.32E+05	7.6
	Mean		5.78E+03	1.17E+04	2.02E+04	1.46E+05	
25	R1	7.0	6.35E+03	1.08E+04	2.69E+04	4.96E+04	7.6
	R2	7.0	5.95E+03	9.12E+03	3.35E+04	6.13E+04	7.5
	R3	7.1	5.73E+03	9.84E+03	1.56E+04	5.01E+04	7.5
	Mean		6.01E+03	9.92E+03	2.54E+04	5.37E+04	
50	R1	7.0	7.52E+03	8.70E+03	3.86E+03	5.19E+03	7.3
	R2	7.0	6.14E+03	9.81E+03	4.96E+03	4.89E+03	7.1
	R3	7.0	5.18E+03	8.78E+03	4.34E+03	5.16E+03	7.1
	Mean		6.28E+03	9.10E+03	4.38E+03	5.08E+03	
100	R1	7.0	5.11E+03	2.38E+03	3.68E+03	6.06E+03	7.2
	R2	7.0	5.10E+03	2.77E+03	2.41E+03	1.40E+03	7.2
	R3	7.0	5.59E+03	2.61E+03	2.10E+03	2.30E+03	7.1
	Mean		5.27E+03	2.59E+03	2.73E+03	3.26E+03	

*Cell densities represent thean number of cells per ml calculated from thean of the cell counts from 3 counts for each of the replicate flasks. R1 – R6: Replicates 1 to 6.

Table 2 : Daily Specific Growth Rates for the Control Cultures in the Definitive Test

		Daily Specific Growth Rate (cells /mL /hour)

		Day 0 - 1	Day 1 - 2	Day 2 - 3
Control	R1	0.041	0.073	0.069
	R2	0.053	0.060	0.068
	R3	0.047	0.050	0.068
	R4	0.045	0.057	0.077
	R5	0.045	0.059	0.075
	R6	0.047	0.068	0.058
	Mean	0.046	0.061	0.069

R1- R6= Replicates 1 to 6

Table 3 : Inhibition of Growth Rate and Yield in the Definitive Test

Nominal concentration (mg/L)		Growth rate (cells /mL/hour)		Yield (cells/mL)	
		0 - 72 h	% Inhibition	0 - 72 h	% Inhibition*
Control	R1	0.060		3.04E+05	
	R2	0.060		2.97E+05	
	R3	0.054		1.98E+05	
	R4	0.059	-	2.72E+05	-
	R5	0.059		2.83E+05	
	R6	0.058		2.51E+05	
	Mean	0.058		2.67E+05	
	SD	0.002		3.91E+04	
6.25	R1	0.062	[7]	3.30E+05	
	R2	0.061	[5]	3.09E+05	
	R3	0.060	[3]	3.07E+05	
	Mean	0.061	[5]	3.15E+05	[18]
	SD	0.001		1.29E+04	
12.5	R1	0.052	10	1.68E+05	
	R2	0.048	17	1.26E+05	
	R3	0.049	16	1.27E+05	
	Mean	0.050	14	1.40E+05	48
	SD	0.002		2.40E+04	
25	R1	0.035	40	4.33E+04	
	R2	0.038	34	5.54E+04	
	R3	0.035	40	4.43E+04	
	Mean	0.036	38	4.77E+04	82
	SD	0.002		6.70E+03	
50	R1	0.004	93	-2.33E+03	
	R2	0.003	95	-1.26E+03	
	R3	0.004	93	-1.60E+01	
	Mean	0.004	94	-1.20E+03	100
	SD	0.001		1.16E+03	

100	R1	0.006	90	9.52E+02	
	R2	-0.015	126	-3.70E+03	
	R3	-0.008	114	-3.29E+03	
	Mean	-0.006	110	-2.01E+03	101
	SD	0.011		2.58E+03	

*In accordance with the OECD test guideline only thean value for yield for each test concentration is calculated

R1- R6= Replicates 1 to 6 SD= Standard Deviation

Table 4 : Concentrations in Aqueous Test Media Samples Determined Using GC Analysis

Sample	Nominal Concentration (mg/l)	Concentration Found (mg/l)	Expressed as a Percentage of the Nominal Concentration (%)
0 hours	Control	<LOQ	-
	6.25	6.63	106
	12.5	13.3	106
	25	24.3	97
	50	50.8	102
	100	105	105
72 hours	Control	<LOQ	-
	6.25	<LOQ	-
	12.5	<LOQ	-
	25	<LOQ	-
	50	<LOQ	-
	100	23.7	24

LOQ = Limit of quantitation

Table 5 : The geometric mean measured test concentrations were determined to be:

Nominal Test Concentration (mg/L)	Geometric Mean Measured Test Concentration (mg/l)	Expressed as a % of the Nominal Test Concentration
6.25	0.79	13
12.5	1.1	9
25	1.5	6
50	2.2	4
100	50	50

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3.3 Chronic toxicity

3.3.1 Chronic toxicity to algae or aquatic plants

See 3.2.3 Algal growth inhibition tests