



**Opinion of the Scientific Panel on Food Additives, Flavourings,
Processing Aids, Materials in Contact with Food and Cosmetics of
the Norwegian Scientific Committee for Food Safety**

Adopted 7 March 2008

**Risk assessment of *N*-ethyl-toluenesulfonamide (NETSA) used as
plasticizer in printing inks on food packaging materials**

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SUMMARY

The Norwegian Food Safety Authority (Mattilsynet) asked the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) to perform a risk assessment of *N*-ethyl-toluenesulfonamide (NETSA) used as plasticizer in printing inks on food packaging materials based on earlier data as well as new data provided. If possible, a tolerable daily intake (TDI) or a tolerable weekly intake (TWI) value for NETSA should be established. The case has been assessed by the Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics (Panel 4).

NETSA is a generic term for the ortho (2-), meta (3-) and para (4-) isomers of *N*-ethyl-toluenesulfonamide, and mixtures of these. In this opinion, the main focus is on the commercial product (CAS no. 8047-99-2), consisting of *N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) and *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7).

In the three available repeated dose toxicity studies of NETSA in rats, early, transient effects on the central nervous system (CNS), such as lethargy and uncoordinated movements, were observed. The overall lowest observed adverse effect level (LOAEL) value for these acute effects on CNS was 18.75 mg/kg bw/day. It should be noted that this value is of similar magnitude as the subchronic LOAEL value. Increased absolute and relative weights of liver and kidneys were observed in a 90-day study, indicating liver and kidney toxicity. The LOAEL value for these subchronic effects was 25 mg/kg bw/day based on significantly increased relative liver weight in female rats.

NETSA was tested for mutagenic potential in two non-guideline bacterial reverse mutation assays. In the first study, NETSA was a mutagen in TA1535 without S9-mix. The second study was inconclusive. NETSA was genotoxic (presumably clastogenic) in the presence of S9-mix in the mouse lymphoma assay. NETSA was clastogenic in human lymphocytes *in vitro* in the presence of S9-mix, and induced a high occurrence of exchanges. There was also an indication that the test product has an aneugenic potential. The clastogenic and aneugenic potential, i.e. the ability to cause structural and numerical chromosomal aberrations, was not confirmed in two *in vivo* micronucleus assays. However, exchanges will not be detected in this micronucleus assay. Therefore, further studies are necessary to clarify the mutagenic and genotoxic potential of NETSA.

Some of the data submitted for evaluation, are from studies using mixtures of ortho and para isomers of NETSA in unknown percentages. Other available data, a.o. QSAR predictions, indicate that the ortho isomer has greater potential for mutagenicity and genotoxicity than the para isomer of NETSA. Therefore, in new studies, the “worst case” mixture of NETSA should be used, i.e. with the highest concentrations of the ortho isomer. If the meta isomer is used in connection with food packaging materials, this isomer should also be tested. The specification/composition of the mixture should be described in detail.

Based on the new submitted data and earlier available data, it is not possible to establish a TDI or TWI value for NETSA. Hence, a conclusion cannot be reached regarding maximum allowable migration levels of NETSA, as requested by the applicant, because the mutagenic and genotoxic potential of NETSA was not determined with certainty.

A migration value of 13 mg/kg food simulant was measured in Norway from cheese films, and a migration limit of 5 mg/kg food was suggested by the applicant. The Panel noted that in spite of possible negative mutagenicity data, when using the EU exposure model, these migration values are incompatible with the submitted data showing acute and subchronic toxicity with LOAELs in the range of 18.75 to 25 mg/kg bw/day.

SAMMENDRAG (In Norwegian)

Mattilsynet bad Vitenskapskomiteen for mattrygghet (VKM) om å gjøre en risikovurdering av *N*-etyl-toluensulfonamid (NETSA) brukt som mykgjørere i trykkfarger på matemballasje ut fra tidligere data og nye fremlagte data. Hvis mulig, skulle også en verdi for tolerabelt daglig inntak (TDI) eller tolerabelt ukentlig inntak (TWI) for NETSA etableres. Saken ble vurdert av Faggruppen for tilsetningsstoffer, aroma, matemballasje og kosmetikk (Faggruppe 4).

NETSA brukes som et fellesnavn for orto- (2-), meta- (3-) and para- (4-)isomerene av *N*-etyl-toluensulfonamid, og blandinger av disse. Denne risikovurderingen fokuserer på det kommersielle produktet (CAS-nr. 8047-99-2), som består av *N*-etyl-2-toluensulfonamid (CAS-nr. 1077-56-1) og *N*-etyl-4-toluensulfonamid (CAS-nr. 80-39-7).

I de tre fremlagte toksisitetsstudiene med gjentatt oral eksponering for NETSA i rotter ble det observert tidlige, forbigående effekter på sentralnervesystemet (CNS), slik som dorskhet og ukoordinerte bevegelser. Det laveste observerte skadelige effekt-nivået (LOAEL) i disse tre studiene for slike akutte effekter på CNS var 18,75 mg/kg kroppsvekt/dag. Denne verdien er av samme størrelsesorden som den subkroniske LOAEL-verdien. Økt absolutt og relativ vekt av lever og nyrer ble observert i en oral 90 dagers studie på rotter, noe som tyder på lever- og nyretoksisitet. Den laveste LOAEL-verdien for subkroniske effekter var 25 mg/kg kroppsvekt/dag basert på signifikant økt relativ levervekt i hunn-rotter.

NETSA ble testet for mutagent potensiale i to mutasjonstester i bakterier, som ikke var utført etter gjeldende retningslinjer. I den første studien var NETSA mutagent i TA1535-stammen uten metabolsk aktivering med S9. Den andre studien førte ikke til en klar konklusjon. NETSA var gentoksisk (antagelig klastogent) med S9 tilstede i muse-lymfomtesten. NETSA var klastogent i humane lymfocytter *in vitro* med S9 tilstede, og induerte en høy forekomst av kromosomutbyttinger. Det var også indikasjoner på at testforbindelsen hadde aneugent potensiale. En potensiell klastogen og aneugen effekt, dvs. evne til å forårsake strukturelle og numeriske kromosomforandringer, ble ikke bekreftet i to *in vivo* mikrokjernetester. Men en slik mikrokjernetest vil ikke kunne detektere kromosomutbyttinger. Det trengs derfor flere studier for å klargjøre det mutagene og gentoksiske potensialet til NETSA.

Noen av dataene som ble sendt inn for vurdering var fra studier med blandinger av orto- og para-isomerene av NETSA i ukjent prosentforhold. Andre tilgjengelige data, bl.a. QSAR-vurderinger, tyder på at orto-isomeren hadde større mutagent og gentoksisk potensiale enn para-isomeren av NETSA. Derfor bør man i nye studier bruke den potensielt mest toksiske blandingen av isomerer av NETSA, dvs. den med høyest konsentrasjon av orto-isomeren. Den eksakte sammensetningen av blandingen som brukes må beskrives i detalj.

På grunnlag av de nye innsendte studiene og tidligere tilgjengelige data er det ikke mulig å fastsette en TDI- eller TWI-verdi for NETSA. Derfor kan det heller ikke konkluderes vedrørende en maksimalt tillatt migrasjon av NETSA, slik søker ønsket, fordi det mutagene og gentoksiske potensialet til NETSA ikke er klarlagt.

En migrasjon på 13 mg/kg simulant ble påvist i Norge fra plastemballasje til ost, og en øvre migrasjonsgrense på 5 mg/kg mat ble foreslått av søker. Faggruppen bemerker at selv om det skulle vise seg at en endelig konklusjon blir at NETSA ikke er mutagent, vil disse migrasjonsverdiene ved bruk av EUs modell for beregning av eksponering fra matkontaktmaterialer ikke være forenlige med de innsendte dataene som viste akutt og subkronisk toksisitet med LOAEL-verdier i området 18,75 til 25 mg/kg kroppsvekt/dag.

LIST OF ACRONYMS

ALAT	-	alanine aminotransferase
ASAT	-	aspartate aminotransferase
bw	-	body weight
CAS	-	Chemical Abstract Service
CNS	-	central nervous system
DMSO	-	dimethyl sulfoxide
EFSA	-	The European Food Safety Authority
EINECS	-	European Inventory of Existing Chemical Substances
FDA	-	U.S. Food and Drug Administration
GLP	-	good laboratory practise
IMM	-	The Institute of Environmental Medicine at Karolinska Institutet, Stockholm, Sweden
IWGT	-	International Workshop on Genotoxicity Testing
LD50	-	lethal dose 50%
LOAEL	-	lowest observed adverse effect level
MOS	-	margin of safety
NETSA	-	<i>N</i> -ethyl-toluenesulfonamide
NOAEL	-	no observed adverse effect level
NTP	-	National Toxicology Program, U.S.A.
OECD	-	The Organization for Economic Co-Operation and Development
PCE	-	polychromatic erythrocytes
QSAR	-	quantitative structure-activity relationship
RTECS	-	The Registry of Toxic Effects of Chemical Substances
SOP	-	standard operating procedure
TDI	-	tolerable daily intake
tk	-	thymidine kinase
TWI	-	tolerable weekly intake
VKM	-	The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet)

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Assessed by

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1 BACKGROUND

The Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics of the Norwegian Scientific Committee for Food Safety (VKM) has earlier performed a risk assessment, dated November 9, 2004, concerning migration of *N*-ethyl-toluenesulfonamide (NETSA) from cheese film to food simulant (1). The main conclusion from this risk assessment was that the lack of toxicological data made it impossible to rule out potential negative health effects. More data was needed to perform a complete risk assessment, leading to establishment of a tolerable daily intake (TDI) value or a tolerable weekly intake (TWI) value. The Norwegian Food Safety Authority (Mattilsynet) has in a letter of March 12, 2007, asked VKM to perform a risk assessment of NETSA where new data is included. The new data was submitted by a producer of NETSA (commercial name Ketjenflex[®] 8), and by the Institute of Environmental Medicine (IMM) at Karolinska Institutet, Stockholm, Sweden, from an experiment with NETSA performed in commission by The National Food Administration (Livsmedelsverket) in Sweden. In this opinion, in addition to the data available for evaluation in 2004 (1), the following new experiments were evaluated:

General toxicity

- Evaluation of the acute oral toxicity of Ketjenflex[®] 8 in the rat (1985). NOTOX 0187/264 (2).
- 14-Day oral range finding study with Ketjenflex[®] 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 442496 (3).
- Allmäntoxicitetsstudie av *N*-etyl-*o,p*-toluen-sulfonamid (NETSA) på råttor vid daglig peroral tillförsel under 28 dagar, TUT-05 Slutrapport, 9/19/06 (In Swedish) (General toxicity study of *N*-ethyl-*o,p*-toluenesulfonamide (NETSA) on rat with daily peroral administration in 28 days) (2006), The Institute of Environmental Medicine (IMM), Karolinska Institutet, Stockholm, Sweden (4).
- Repeated dose 90-day oral toxicity study with Ketjenflex[®] 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 441282 (5).

Mutagenicity/genotoxicity

- Ames/Salmonella mutagenicity assay of Santicizer[®] 8 Plasticizer (1984). Report No. MSL-3660, Job/Project No. ML-83-198/830075 (6).
- Evaluation of the mutagenic activity of Ketjenflex[®] 8 (NETSA) in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat) (2005). NOTOX Project 441315 (7).
- Evaluation of the ability of Ketjenflex[®] 8 (NETSA) to induce chromosome aberrations in cultured peripheral human lymphocytes (with repeat experiment) (2006). NOTOX Project 441304 (8).
- Micronucleus test in bone marrow cells of the rat with Ketjenflex[®] 8 (NETSA) (2006). NOTOX Project 452824 (9).
- Micronucleus evaluation in bone marrow from some animals from the repeated dose 90-day oral toxicity study with Ketjenflex[®] 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 441282 (10).

2 TERMS OF REFERENCE

The Norwegian Food Safety Authority (Mattilsynet) has in a letter of March 12, 2007, asked VKM, the Scientific Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics, to

- perform a risk assessment of NETSA based on the new data as well as earlier data, and
- establish a TDI or TWI value, if possible.

3 OPINION

Most of the new experiments under evaluation in this opinion are with the commercial NETSA product, Ketjenflex[®] 8. The production of Ketjenflex[®] 8 leads to a mixture (CAS no. 8047-99-2), consisting of *N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) and *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7). In most of the submitted experimental reports, the exact percentages of the two compounds are not stated. However, according to the product data sheet of Ketjenflex[®] 8 (11), it is a mixture of approximately 70% *N*-ethyl-2-toluenesulfonamide and 30% *N*-ethyl-4-toluenesulfonamide. The composition of Ketjenflex[®] 8 may according to the safety data sheet (12) vary from 40-75% *N*-ethyl-2-toluenesulfonamide and 25-60% *N*-ethyl-4-toluenesulfonamide. In one of the newly submitted experiments (6), the old commercial name Santicizer[®] 8 is used for this mixture (CAS no. 8047-99-2). The exact information about the product, including the batch number and purity, is specified under the individual experiments if stated in the submitted experimental reports.

Some data were found for the individual isomers of NETSA, having other CAS numbers than 8047-99-2. These data are included in the Appendix.

3.1 Chemical and physical specifications

3.1.1 Chemical names

N-ethyl-2-toluenesulfonamide (ortho isomer)

N-ethyl-3-toluenesulfonamide (meta isomer)

N-ethyl-4-toluenesulfonamide (para isomer)

2-toluenesulfonamide, *N*-ethyl- (ortho isomer)

3-toluenesulfonamide, *N*-ethyl- (meta isomer)

4-toluenesulfonamide, *N*-ethyl- (para isomer)

N-ethyl-2-methylbenzenesulfonamide (ortho isomer)

N-ethyl-3-methylbenzenesulfonamide (meta isomer)

N-ethyl-4-methylbenzenesulfonamide (para isomer)

Benzenesulfonamide, *N*-ethyl-2-methyl- (ortho isomer)

Benzenesulfonamide, *N*-ethyl-3-methyl- (meta isomer)

Benzenesulfonamide, *N*-ethyl-4-methyl- (para isomer)

3.1.2 Abbreviations and trade names

NETSA

Ketjenflex[®] 8, Santicizer[®] 8

3.1.3 CAS numbers and EINECS numbers

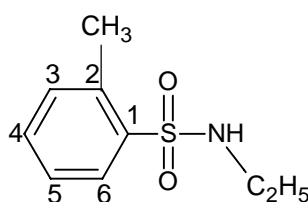
The mixture with CAS no. 8047-99-2 and EINECS no. 232-465-2 consists of *N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) and *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7), and is described in the main part of this opinion.

N-ethyl-2-toluenesulfonamide has CAS no. 1077-56-1 and EINECS no. 214-073-3, or CAS no. 80-37-2 (included in the Appendix).

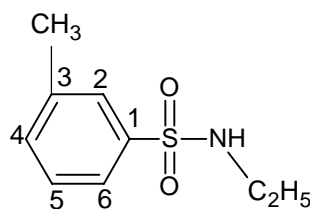
N-ethyl-4-toluenesulfonamide has CAS no. 80-39-7 and EINECS no. 201-275-1 (included in the Appendix).

N-ethyl-3-toluenesulfonamide has CAS no. 66898-18-8 (no toxicity data found).

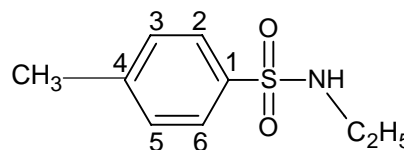
3.1.4 Structural formulas



N-ethyl-2-toluenesulfonamide
N-ethyl-*o*-toluenesulfonamide



N-ethyl-3-toluenesulfonamide
N-ethyl-*m*-toluenesulfonamide



N-ethyl-4-toluenesulfonamide
N-ethyl-*p*-toluenesulfonamide

3.1.5 Empirical formula

C₉H₁₃NO₂S

3.1.6 Physical form

Light yellow viscous liquid

3.1.7 Molecular weight

199.3

3.1.8 Purity and batch number

Purity from 89.0-99.6%. See under individual experiments.

3.1.9 Additional physical and chemical specifications

Boiling point:	196°C (1.4 kPa)
Flash point:	174°C (Cleveland open cup)
Vapour pressure:	<130 Pa (150°C)
Density:	1200 kg/m ³
Viscosity:	250-400 mPa·s (at 25°C)

3.1.10 Partition coefficient (log P_{ow})

The octanol/water partition coefficient for Ketjenflex[®] 8 as log P_{ow} was reported to be 1.8 (13) determined according to the Organization for Economic Co-Operation and Development (OECD) Guidelines for the Testing of Chemicals, Test No. 117: Partition Coefficient (n-octanol/water), High Performance Liquid Chromatography (HPLC) Method (14) (adopted 13 April 2004), indicating low accumulative potential in the human body (15).

3.1.11 Solubility

Practically insoluble in water. Soluble in organic solvents except petroleum hydrocarbons (2). Dimethyl sulfoxide (DMSO), propylene glycol or corn oil were used as solvents (vehicles) as stated under the individual experiments.

3.1.12 Stability

According to the submitted experimental reports, it is stable when stored at room temperature in the dark. Stability in DMSO: not indicated in the experimental reports. Stability in propylene glycol: at least 8 days (5, 9).

3.2 Function and uses

Ketjenflex[®] 8 is a plasticizer and adhesion promoter with high thermal stability. The product is highly polar, and plasticizes resins such as nylon and other polyamides, shellac, cellulose acetate, zein and other protein materials. Its applications include use in polyvinyl acetate emulsion adhesives, nitrocellulose lacquers, cellulose acetate compositions, synthetic polyamides, epoxy adhesives, nail lacquers, printing inks and textile adhesives (11).

3.3 Hazard identification and characterization

3.3.1 Acute toxicity

The lethal dose 50% (LD50) of NETSA (CAS no. 8047-99-2) was reported in The Registry of Toxic Effects of Chemical Substances (RTECS) to be 2.25 g/kg body weight (bw) in rats after oral exposure, with the effects gastritis and hemorrhage (16). The lowest lethal dose in rabbit after oral exposure was 2.0 g/kg bw with the symptoms gastritis and normocytic anemia. Lethal dose after dermal exposure in rabbit was >10.0 g/kg bw.

Evaluation of the acute oral toxicity of Ketjenflex[®] 8 in the rat (1985). NOTOX 0187/264 (2)

Guidelines:	The OECD Guideline for the Testing of Chemicals No. 401: Acute Oral Toxicity (14) (adopted 12 May 1981)
Species/Strain:	Wistar rats (SPF quality, randomly bred)
Group size:	5 males and 5 females
Test substance:	Ketjenflex [®] 8 (<i>N</i> -substituted toluenesulfonamide, CAS no. not stated)
Batch:	Not stated
Purity:	Impurity: approximately 10% o/p toluenesulfonamide
Dose levels:	4.2, 7.4 or 13.0 g/kg bw
Route:	Oral, gavage
Exposure:	A single administration and a 14-day observation period
GLP:	Not stated

Results

In the low dose group, 2 of 10 rats died, in the medium dose group, 7 of 10 rats died, and in the high dose group, 10 of 10 rats died, all within 3 days of dosing. There was insufficient data to assess sex-related effects. Signs of toxicity were apathy, reduced locomotive activity, and laboured breathing, tremors, reduced or bloody fecal excretion. For surviving animals these signs were reversible, since as of day 4 no more abnormalities were observed during the 14-day observation period. Animals found dead showed body weight loss, whereas surviving animals showed normal weekly body weight gain. Macroscopic observations at autopsy of animals found dead after day 1 revealed petechiae or hemorrhages of the stomach wall, superficial liver necrosis in areas adjacent to the stomach and marked hematuria. These findings were indicative of stomach perforation and damage of the urogenital tract. Animals found dead on day 1 and animals sacrificed at the end of the study showed no treatment related gross abnormalities.

Conclusions from study authors

The LD50 value for the sexes combined amounted to approximately 5.8 g/kg bw with a 95% confidence limit (4.4 – 7.4 g/kg bw).

General comments from VKM on acute toxicity

Ketjenflex[®] 8 has low acute toxicity.

3.3.2 Irritation and sensitization

According to RTECS, NETSA (CAS no. 8047-99-2) may cause irritation to the skin and the respiratory tract, and is a mild eye irritant (16).

General comments from VKM on irritation and sensitisation

Ketjenflex[®] 8 may cause irritation to the eyes, skin and respiratory tract. No data with the CAS no. 8047-99-2 was found on sensitisation.

3.3.3 Repeated dose toxicity

14-Day oral range finding study with Ketjenflex[®] 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 442496 (3)

Guidelines:	Based upon, but not fully compliant with the OECD Guideline for the Testing of Chemicals, No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents (adopted July 27, 1995) (14), and Commission Directive 96/54/EC of 30 July 1996, Annex IV D B.7 Repeated dose (28 days) toxicity (oral) (17), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)
Species/Strain:	Rat, Wistar Crl:(WI) BR (outbred, SPF quality), both sexes, six weeks old
Group size:	3 males and 3 females per group
Test substance:	Ketjenflex [®] 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Dose levels:	0, 150.0, 450.0 and 1000.0 mg/kg bw/day
Route:	Oral, by daily gavage for 14 days
Vehicle:	Polypropylene glycol
GLP:	In compliance

The following parameters were evaluated: clinical signs daily, body weight and food consumption weekly, clinical pathology, macroscopy and organ weights at termination. No tissues were fixed.

Results

At 450.0 and 1000.0 mg/kg bw/day clinical signs consisted of lethargy, uncoordinated movements, abnormal gait and/or flat posture after dosing. At 150.0 mg/kg bw/day, only lethargy and/or uncoordinated movements were incidentally observed after dosing.

The higher liver weights and liver to body weight ratios in females at 450.0 and 1000.0 mg/kg bw/day were associated with increased alanine aminotransferase (ALAT) activity levels and cholesterol levels. Higher ALAT activity levels in males at 1000.0 mg/kg bw/day occurred in the absence of clear changes in liver weight at this dose. Dose-related trends in increased liver weights and liver to body weight ratios were seen in males over the dose groups.

There were no changes in body weight and food consumption, or alterations during hematological investigations and macroscopic examination that were considered to be an effect of treatment.

Conclusions from study authors

Based on the results of this study, dose levels for the 90-day oral toxicity study in rats with Ketjenflex[®] 8 were selected to be 25.0, 100.0 and 400.0 mg/kg bw/day.

Comments from VKM

In this study, all three doses showed central nervous system (CNS) effects. The lowest observed adverse effect level (LOAEL) was 150.0 mg/kg bw/day.

General toxicity study of *N*-ethyl-*o,p*-toluenesulfonamide (NETSA) on rat with daily peroral administration in 28 days (2006), The Institute of Environmental Medicine (IMM), Karolinska Institutet, Stockholm, Sweden (4)

Guidelines:	The OECD Guideline for the Testing of Chemicals, No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents (14)
Species/Strain:	Rat, Sprague-Dawley, both sexes, five weeks old
Group size:	Initial dose-finding study: 2 rats per group (sex not specified) Main study: 5 males and 5 females per group
Test substance:	NETSA from Scientific Polymer Products (Ontario, N.Y., U.S.A.) consisting of 60% <i>N</i> -ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) and 40% <i>N</i> -ethyl-4-toluenesulfonamide (CAS no. 80-39-7)
Batch:	Not stated
Purity:	Not stated
Dose levels:	Initial dose-finding study: 0, 187.5, 375.0, 750.0 and 1500.0 mg/kg bw/day Main study: 0, 18.75, 75.0 and 300.0 mg/kg bw/day
Route:	Oral, by daily gavage for 5 days (initial dose-finding study) or 28 days, except on one day during the last week before termination when urine was sampled (main study)
Vehicle:	Corn oil
Negative control:	Tap water
GLP:	In compliance, also with Standard Operating Procedures (SOPs) at the Institute of Environmental Medicine (IMM) at Karolinska Institutet

Initial dose-finding study

On the first day, a strong effect on the CNS was seen in the three highest dose groups, as a strong sedative effect, difficulty with breathing and general illness. The rats were dragging their hind-legs behind them before they fell asleep. Four of six animals showed reversion of these effects within the first day, whereas one rat in each of the two highest dose groups died. The two remaining rats from these groups were used to make up a new high dose group, and the doses were lowered to 200.0, 50.0 and 12.5 mg/kg bw/day. After these doses, no effects were observed. On the third day, the doses were increased to 300.0, 100.0 and 33.0 mg/kg bw/day. Again no effects were observed, and the highest dose was increased to 400.0 mg/kg bw/day.

Main study

In the main study, body weights and food consumption were recorded weekly. General observations were done daily, more detailed observations were recorded weekly, and neurological observations of motor activity, temperament, reflexes, hearing etc. were done during the week of acclimatisation and during week 4 of the study.

In addition to various test parameters for urine, blood samples were taken from all animals on the day of termination. Various hematological parameters, blood chemistry and differential counting were examined. At autopsy, organ weights were recorded and relative organ weights (divided by body weight) were calculated. Histopathology was performed on the organs.

Results

The female rats in the high dose group had significantly lower body weight gain compared to the rats in the vehicle and tap water groups, whereas this effect on body weight gain was not

observed in the male rats. Female rats in the vehicle group and all NETSA groups had lower food consumption than the tap water group decreasing throughout the study, and again, this was not seen in the male rats.

Of the studied urine parameters, the only statistical significant difference between the treatment groups was a decrease in urine osmolality in the females in the high dose group compared with the vehicle group. Since there were no significant changes in glucose or protein values in the urine samples, the decrease in osmolality was not likely to be caused by disturbed water resorption in the kidneys. There were no significant differences between the dose groups of both gender in the clinical-chemical blood analyses, although some significant differences were detected in one gender separately. However, no histopathological findings or other observations were done that could explain these differences. Some significant differences between treatments groups were observed in total weights or relative weights of liver, spleen, kidney, testis and thymus, but the differences did not show any dose-response. Neither the histopathological or pathological findings showed any dose-related effects.

Initially during the study, as well as during the dose-finding study, rats from the high and medium dose groups appeared drowsy and unwell ten minutes after the administration of NETSA. In addition, the rats from the other treatment group also were less active after the administration. However, the effect diminished after 3 hours, and no obvious chronic effects were recorded after 28 days. According to the authors, this type of acute and temporary effect on the activity and consciousness of the rats was probably due to a negative influence on the CNS.

Conclusions and comments by VKM

Both in the initial dose-finding study and in the main study, transient effects on CNS were observed at all doses. A no observed adverse effect level (NOAEL) could not be determined for these acute effects. The LOAEL was 18.75 mg/kg bw/day for acute effects on CNS. A significant reduction in body weight gain was found in female rats in the high dose group (300.0 mg/kg bw) in the main study.

Repeated dose 90-day oral toxicity study with Ketjenflex[®] 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 441282 (5)

Guidelines:	The OECD Guideline for the Testing of Chemicals, No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents (adopted 21 September 1998) (14), and Commission Directive 2001/59/EC of 6 August 2001, Annex 5D, B.26. Subchronic oral toxicity test, repeated dose 90-day oral toxicity study in rodents (19), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)
Species/Strain:	Rat, Wistar Crl:(WI) BR (outbred, SPF quality), six weeks old
Group size:	10 males and 10 females per group
Test substance:	Ketjenflex [®] 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Dose levels:	0, 25.0, 100.0 and 400.0 mg/kg bw/day (based on the 14-day oral range finding study described above (3))
Route:	Oral, by daily gavage for at least 90 days, 7 days per week

Vehicle: Propylene glycol (stability of the test substance in the vehicle was confirmed for at least 8 days)
GLP: In compliance

The following parameters were evaluated: clinical signs, functional observations, body weight, food consumption and ophthalmoscopy. At termination, clinical pathology, macroscopy, organ weights and histopathology on a selection of tissues were performed.

Results

Clinical signs observed after dosing, including lethargy, flat posture and uncoordinated movements, showed a dose-related occurrence with regard to incidence and time of appearance. In addition, it was frequently noted that animals secluded from their cage mates after dosing. Functional observations, motor activity measurements and histopathological assessment of neuronal tissues revealed no abnormalities that would support these clinical observations. The symptoms were of a minor and temporary nature, i.e. occurring only a short period (1 hour) after dosing, and not overtly and consistently present within the same dose group for the duration of treatment. With the exception of these signs, animals appeared in a good health throughout the study period.

In most cages containing rats given 400.0 mg/kg bw/day, dry feces was recorded on most days from week 4 of treatment and onwards. In one instance, this was recorded as pale feces for high dose females on day 22.

Salivation seen after dosing among animals of the 400.0 and 100.0 mg/kg bw/day dose groups is often noted in rats of this strain and age following oral gavage. Considering the nature and minor severity of the effect and its time of occurrence (i.e. after dosing), this was considered to be a physiological response rather than a sign of systemic toxicity.

Cortical hyaline droplets representing $\alpha_2\mu$ globulin, a normal protein in male rats which undergoes re-absorption in the proximal cortical tubules, were recorded at increased incidence and severity in the kidneys of males given 400.0 mg/kg bw/day. Kidney to body weight ratios were slightly, but significantly, increased for these males. The hyaline droplets were accompanied by an increase in the incidence of minor degrees of hyaline casts, and increased incidence and severity of corticomedullary tubular basophilia. These latter findings were considered as indicators of renal tubular damage due to the increase in hyaline droplets. Although the incidence of hyaline droplets was increased also at 100.0 mg/kg bw/day compared to the controls, the alteration was not accompanied by an increase in the abovementioned indicators of tubular damage (hyaline casts and corticomedullary tubular basophilia), and the severity grades of minimal or slight were within the range which may be seen in untreated male rats. This specific male response is not observed in normal female rats, or in higher species of either sex, including humans.

In females, dose-related increases both in absolute and relative kidney weight were observed, although the increase was statistically significant only after 400.0 mg/kg bw for absolute kidney weight, and after 100.0 and 400.0 mg/kg bw/day for relative kidney weight. No toxicological significance was ascribed to the higher absolute and/or relative kidney weights of females at 100.0 and 400.0 mg/kg bw/day, since no morphological correlate was found.

In females, significantly increased absolute liver weights were observed after 100.0 and 400.0 mg/kg bw/day, and dose-related significant increases in relative liver weight were seen after

25.0, 100.0 and 400.0 mg/kg bw/day. In males, there was a dose-related increase in relative liver weight, although the increase in absolute and relative liver weight was statistically significant only after 400.0 mg/kg bw. In females given 400.0 mg/kg bw/day, diffuse midzonal/centrilobular hypertrophy in the liver was present in seven cases at minor degrees of severity. In the other groups, the increased absolute or relative liver weight occurred in the absence of any morphological correlates or microscopic evidence of any degenerative changes that would be indicative of hepatocytotoxicity. Therefore, these alterations were considered by the study authors to be an adaptive physiological response following xenobiotics administration. The higher total protein, albumin and cholesterol levels in males and females given 400.0 mg/kg bw/day were considered to be related to these liver findings, and means of these parameters were largely within the normal range to be expected for rats of this strain and age.

The slightly higher mean potassium and calcium levels in females given 400.0 mg/kg bw/day remained within the normal range expected for rats of this strain and age, and corresponding morphological evidence of organ damage was absent in this sex. Therefore, these clinical biochemistry findings were considered not to reflect changes considered as adverse in toxicological sense by the study authors.

Body weights and body weight gains of treated animals remained in the same range as the vehicle controls over the study period. There were no changes in food consumption, ophthalmoscopy, functional observations, hematological investigations or macroscopic examinations that were considered to be treatment-related.

Conclusions from study authors

Although a relation to treatment with the test substance could not be excluded, the transient effects on CNS were regarded to be of no toxicological significance. Histopathological findings in male kidneys after 400.0 mg/kg bw/day were considered to represent an adverse effect of the test substance. Findings at 100.0 or 25.0 mg/kg bw/day occurred in the absence of supportive functional or morphological disturbances indicative of organ dysfunction. The study authors therefore concluded that a NOAEL value of 100.0 mg/kg bw/day was established on the basis of these results for Ketjenflex[®] 8 in rats. It was also concluded that Ketjenflex[®] 8 was not clastogenic based on evaluation of micronuclei from the bone marrow of femurs from some of these animals (described separately below (10)).

Comments from VKM

VKM disagrees with the study authors that the observed transient effects on CNS, i.e. lethargy, uncoordinated movements etc., were of no toxicological significance. These effects were dose-related with regard to incidence and time of appearance, and therefore most likely treatment-related. VKM considers these effects to be of toxicological significance.

VKM considers the effects on liver weight more relevant for setting a NOAEL value for subchronic effects than the effects on kidneys in male rats. The absolute liver weight was significantly increased after exposure to the two highest doses in females and to the highest dose in males. A significantly increased relative liver weight was seen only in the highest dose in males, whereas in females this was observed in all three dose groups and there was a clear dose-reponse in this effect. Despite microscopic findings of hepatic midzonal/centrilobular hypertrophy only in the highest dose groups in females, VKM concludes that the LOAEL was 25.0 mg/kg bw in females based on significantly increased relative liver weight, indicating possible toxic effects on the liver. A toxic effect on the liver

was also indicated by significantly reduced levels of aspartate aminotransferase (ASAT) in female rats given the two highest doses.

General comments from VKM on repeated dose toxicity

In all three repeated dose toxicity studies of NETSA in rats, early, transient effects on CNS, i.e. lethargy, uncoordinated movements etc., were observed. The overall LOAEL value for these acute effects on CNS were 18.75 mg/kg bw/day. It should be noted that this value is of similar magnitude as the subchronic LOAEL value. Increased absolute and relative weights of liver and kidneys were observed in the 90-day study, indicating liver and kidney toxicity. The LOAEL value for subchronic effects was 25.0 mg/kg bw/day based on significantly increased relative liver weight in female rats.

3.3.4 Chronic (>12 months) toxicity

No data found.

3.3.5 Mutagenicity/genotoxicity

3.3.5.1 *In vitro*

Bacterial gene mutation assay

Ames/Salmonella mutagenicity assay performed by The National Toxicology Program (NTP) in U.S.A. (20)

Guidelines:	Standard NTP protocol
Species/Strain:	<i>Salmonella typhimurium</i> , TA100, TA1535, TA97 and TA98
Replicates:	One assay. Replicates not stated
Assay conditions:	Preincubation assay, both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and male Syrian hamster liver S9
Test substance:	Mixture of <i>N</i> -ethyl-2-toluenesulfonamide and <i>N</i> -ethyl-4-toluenesulfonamide (CAS no. 8047-99-2)
Batch:	Not stated
Purity:	Not stated
Concentrations:	33.0, 100.0, 333.0, 667.0 (TA1535 only, -S9), 1000.0, 1200.0 (TA1535 only, -S9), 2000.0 µg/plate
Solvent:	DMSO
GLP:	Not stated

In 1984, a mixture of *N*-ethyl-2-toluenesulfonamide and *N*-ethyl-4-toluenesulfonamide (CAS no. 8047-99-2) was evaluated by NTP. The product was tested in TA100, TA1535, TA97 and TA98 both with and without S9-mix (5% rat liver, 10% rat liver, 5% hamster liver and 10% hamster liver). Raw data was not shown, only the mean and SD.

Results

The product (CAS no. 8047-99-2) was negative in TA97, TA98 and TA100 both with and without S9-mix, and in TA1535 with S9-mix. In TA1535 without S9-mix, the product

induced a concentration-related and reproducible (in two experiments) increase in revertant colonies.

Conclusions from study authors

The overall results were that the mixture of *N*-ethyl-2-toluenesulfonamide and *N*-ethyl-4-toluenesulfonamide (CAS no. 8047-99-2) was a weakly positive mutagen in bacteria.

Comments from VKM

According to the applicant, the NTP results are disputable, because when using the OECD criteria for the evaluation of the marginally increased values, classification would be negative. VKM does not agree with this statement. In addition, there is some indication that the chemical structure has an implication on the mutagenic effect, since the mixture of the ortho and para isomers (CAS no. 8047-99-2) was weakly positive, but not the para isomer alone (see Appendix for an experiment showing negative results with *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7) in Ames/Salmonella mutagenicity assay).

Ames/Salmonella mutagenicity assay of Santicizer[®] 8 Plasticizer (1984). Report No. MSL-3660, Job/Project No. ML-83-198/830075 (6)

Guidelines:	Not stated
Species/Strain:	<i>Salmonella typhimurium</i> , TA98, TA100, TA1535 and TA1537
Replicates:	Triplicate plates for each microsome/concentration level combination in 3 independent experiments with TA100 without S9-mix, in two experiments with TA 100 with S9-mix, and in only one experiment with the other strains with and without S9-mix
Assay conditions:	Plate incorporation test, both in the presence or absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9-mix. At least 48 hours incubation at 37°C
Test substance:	Santicizer [®] 8 Plasticizer (CAS no. 8047-99-2)
Batch:	Laboratory sample no T830063
Purity:	89%
Concentrations:	Experiment 1: 0.01, 0.04, 0.20, 1.00, 3.00, 10.00 mg/plate in TA98, TA100, TA1535, TA1537 with S9-mix (ref. 6, Table 4) and without (ref. 6, Table 5) Experiment 2: 0.01, 0.04, 0.20, 1.00, 3.00, 10.00 mg/plate in TA 100 with S9-mix (ref. 6, Table 6) Experiment 3: 1.50, 3.00, 4.50 mg/plate in TA100 without S9-mix (ref. 6, Table 7) Experiment 4: 1.50, 3.00, 4.50 mg/plate in TA100 without S9-mix (ref. 6, Table 8)
Solvent:	DMSO
GLP:	Not stated

Santicizer[®] 8 Plasticizer (old commercial name for NETSA) dissolved in DMSO was tested for mutagenicity in the reverse mutation assay in bacteria. The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were exposed to the test substance at concentrations ranging from 0.01 mg/plate to 10.00 mg/plate with and without Aroclor induced rat liver S9-mix. Test concentrations were selected based on the results obtained in a

pre-experiment with tester strain TA100 with and without S9-mix. Appropriate positive and negative (DMSO) controls were included.

Results

In the preliminary toxicity test with TA100, it was stated that the highest concentration of 10.00 mg/plate was toxic, whereas the lower concentrations tested, 3.00, 1.00, 0.20, 0.04 and 0.01 mg/plate, were not toxic. However, it was not mentioned how the toxicity was measured. In the first experiment with S9-mix, toxicity was observed at 3.0 mg/plate and above, evident as a reduction in the number of revertants, in TA100, TA1535 and TA1537 (ref. 6, Table 4). Ten mg/plate was toxic to all strains with S9-mix. Without S9-mix, toxicity was observed at 10.00 mg/plate in TA100, TA1535 and TA1537 (ref. 6, Table 5). In the first experiment, there was a concentration-related increase in revertant colonies in TA100 without S9-mix up to 1.0 mg/plate, hereafter the revertants reached a plateau, which could be due to toxicity (ref. 6, Table 5). No genotoxic effect was observed in the other tester strains either with or without S9-mix (ref. 6, Tables 4 and 5). Although, toxic effect was observed in the first experiment, the same concentrations were used in the second experiment with TA100 with S9-mix (ref. 6, Table 6). The negative result in TA100 with S9-mix from the first experiment was confirmed in the second experiment. However, toxicity was observed at the highest concentrations. In the third experiment with TA100 without S9, only three concentrations were tested (ref. 6, Table 7). At the lowest concentration (1.50 mg/plate), there was a 1.7-fold increase in revertants compared to non-solvent control, and at the two highest concentrations there was a decrease in revertant colonies, which might be due to toxicity. The results from the third experiment were confirmed in a fourth experiment with TA100 without S9-mix (ref. 6, Table 8).

Conclusions from study authors

It was concluded by the study authors that Santicizer[®] 8 Plasticizer was not mutagenic in bacteria.

Comments from VKM

It was not clear from the study protocol how toxicity was measured (reduction in revertants and/or background lawn). The highest concentration tested (10.00 mg/plate) is above the recommended concentration of 5.00 mg/plate (14). The strict criteria for a positive response set by the study authors (three consecutive levels with revertants per plate statistically greater than controls ($p < 0.01$) with a significant dose response ($p < 0.01$)), are not in accordance with the criteria set in OECD guideline 471: Bacterial Reverse Mutation Test (14), i.e. a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation. Since very few non-toxic concentrations were tested, especially with S9-mix, no conclusions can be drawn from this experiment. However, a weak mutagenic potential was suggested in TA 100 without metabolic activation in this study.

Mammalian cell gene mutation test in mouse lymphoma cells (tk locus)

Evaluation of the mutagenic activity of Ketjenflex[®] 8 (NETSA) in an *in vitro* mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat) (2005). NOTOX Project 441315 (7)

Guidelines:	The OECD Guideline for the Testing of Chemicals, No. 476: <i>In Vitro</i> Mammalian Cell Gene Mutation Test (adopted July 21, 1997) (14), and Commission Directive 2000/32/EC of 19 May 2000, Annex 4E, B.17. Mutagenicity – <i>In vitro</i> mammalian cell gene mutation test (21), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)
Species/Strain:	Mouse lymphoma cell line L5178Y (thymidine kinase (<i>tk</i>) locus for trifluorothymidine (TFT) resistance
Replicates:	Single cultures in two independent experiments, and one repeat experiment with S9-mix
Metabolic activation:	Phenobarbital- and β -naphthoflavone-induced male Wistar rat liver S9-mix
Test substance:	Ketjenflex [®] 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Concentrations:	Exp. 1 -S9: 100, 250, 400, 500, 600, 700, 800 μ g/ml Exp. 1 +S9: 250, 500, 600, 700, 800, 850, 900 μ g/ml Exp. 2 -S9: 100, 250, 400, 500, 550, 600, 650, 700 μ g/ml Exp. 2 +S9: 400, 500, 550, 600, 650, 700, 750, 850 μ g/ml Exp. 3 +S9: 500, 600, 700, 735, 770, 800, 835, 870 μ g/ml
Treatments:	Experiment 1: with 8% S9-mix and without S9-mix, both 3 hours treatment Experiment 2: with 12% S9-mix, 3 hours treatment and without S9-mix, 24 hours treatment Experiment 3: with 12% S9-mix, 3 hours treatment All experiments: 2 days expression period
Solvent:	DMSO
GLP:	In compliance

The test substance was examined for its genotoxic (mutagenic and/or clastogenic) potential in the L5178Y *tk*^{+/-} mouse lymphoma test in the absence and presence of metabolic activation. A range-finding test (pre-test on toxicity, measuring relative suspension growth), two independent mutagenicity experiments, and a repeat experiment with S9-mix, were carried out. In the pre-test, concentrations up to the maximum recommended concentration (10 mM) were tested. The concentrations selected for the main experiments were based on the results of the range-finding experiment. One single culture with 7 (1. experiment) and 8 (2. and 3. experiment) concentrations was investigated. Mutant frequency and cell survival (measured as cloning efficiency) were determined in parallel, and the ratio of small versus large colonies was calculated. Positive controls (methyl methane sulfonate without S9-mix and cyclophosphamide with S9-mix) and negative controls (DMSO) were included.

Results

In the first experiment, concentrations of Ketjenflex[®] 8 up to 800 and 900 μ g/ml in the absence and presence of 8% (v/v) S9-mix, respectively, were used. Ketjenflex[®] 8 was tested

up to cytotoxic levels of 84% and 92% in the absence and presence of S9-mix, respectively. In the absence of S9-mix, Ketjenflex[®] 8 did not induce a significant increase in mutation frequency. In the presence of S9-mix, Ketjenflex[®] 8 showed fluctuations in the mutation frequency above the laboratory historical control data range, but no concentration-response relationship was observed, and the maximum increase in mutation frequency was 1.8 compared to the solvent control. The highest induced mutation frequency was 98 at the 600 µg/ml concentration, which is below the International Workshop on Genotoxicity Testing (IWGT) acceptance criteria of 126 for a biological significant response (22, 23).

In the second experiment, concentrations of Ketjenflex[®] 8 up to 700 and 850 µg/ml in the absence and presence of 12% (v/v) S9-mix, respectively, were used. Ketjenflex[®] 8 was tested up to cytotoxic levels of 89% and 90% in the absence and presence of S9-mix, respectively. In the absence of S9-mix, Ketjenflex[®] 8 did not induce a significant increase in mutation frequency. In the presence of S9-mix, Ketjenflex[®] 8 induced up to 3.2-fold increase in mutation frequency at the *tk*-locus. The highest induced mutation frequency was 180 at the second highest concentration (750 µg/ml), which is well above the IWGT acceptance criteria of 126 for a biological significant response (22, 23). In addition, there is some concentration-response relationship between approximately 600 and 800 µg/ml. There was an increase in both small and large colonies, but especially in small colonies.

To verify the results obtained in the second experiment, a third experiment was performed. Ketjenflex[®] 8 was tested in concentrations up to 870 µg/ml in the presence of 12% (v/v) S9-mix. Ketjenflex[®] 8 was tested up to cytotoxic levels of 88%. Ketjenflex[®] 8 now induced up to 2.0-fold increase in mutation frequency at the *tk*-locus, and the dose response relationship between approximately 600 and 800 µg/ml was confirmed in this experiment. The highest induced mutant frequency was 124 at 735 µg/ml. The increase was mainly in small colonies, indicating that the product has a clastogenic potential.

Conclusions from study authors

It was concluded by the study authors that Ketjenflex[®] 8 was not mutagenic in the mouse lymphoma L5178Y test system in the absence of S9-mix and equivocally mutagenic in the presence of S9-mix under the experimental conditions tested.

Comments from VKM

It is the opinion of VKM that Ketjenflex[®] 8 was genotoxic in the presence of S9-mix, and that the optimal concentration is 12% S9 in the S9-mix. Although there was not a clear concentration-related response, the positive response at 750 µg/ml in the 2. experiment was confirmed in the 3. experiment at 735 µg/ml, and the concentration-response relationship was very similar in the 2. and 3. experiment. The increase in mainly small colonies indicates that Ketjenflex[®] 8 has clastogenic potential.

Chromosomal aberration assay

Evaluation of the ability of Ketjenflex[®] 8 (NETSA) to induce chromosome aberrations in cultured peripheral human lymphocytes (with repeat experiment) (2006). NOTOX Project 441304 (8)

Guidelines:	The OECD Guideline for the Testing of Chemicals, No. 473: <i>In Vitro</i> Mammalian Chromosome Aberration Test (adopted July 21, 1997) (14), and Commission Directive 2000/32/EC of 19 May 2000, Annex 4A, B.10. Mutagenicity – <i>In vitro</i> mammalian chromosome aberration test (23), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)
Species/Strain:	Human peripheral lymphocytes
Replicates:	Duplicate cultures in two independent experiments
Metabolic activation:	Phenobarbital- and β -naphthoflavone-induced male Wistar rat liver S9-mix
Test substance:	Ketjenflex [®] 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Concentrations:	Exp. 1 –S9: 1200, 1400, 1600 μ g/ml (3 hours exposure, 24 hours fixation) Exp. 1 +S9: 1000, 1200, 1400 μ g/ml (3 hours exposure, 24 hours fixation) Exp. 2 –S9: 200, 400, 500 μ g/ml (24 hours exposure, 24 hours fixation) and 100, 300, 500 μ g/ml (48 hours exposure, 48 hours fixation) Exp. 2 +S9: 1000, 1300, 1600 μ g/ml (3 hours exposure, 48 hours fixation)
Solvent:	DMSO
GLP:	In compliance

Ketjenflex[®] 8 was investigated in human peripheral lymphocytes for clastogenic potential in the presence and absence of phenobarbital- and β -naphthoflavone-stimulated rat liver S9-mix. A pre-test on cell growth inhibition with 3 hours, 24 hours and 48 hours in the absence of S9-mix, and 3 hours in the presence of S9-mix, using concentrations between 33 and 1993 μ g/ml (up to 10 mM) was performed in order to determine the toxicity of the test substance. The highest concentration in the main tests was based on this pre-test. Positive controls (mitomycin C without S9-mix and cyclophosphamide with S9-mix) and negative controls (DMSO) were included.

Results

In the first cytogenetic assay, Ketjenflex[®] 8 was tested up to 1600 μ g/ml for 3 hours exposure with a 24 hours fixation time in the absence of S9-mix. Ketjenflex[®] 8 precipitated in the culture medium at this dose level and appropriate toxicity was reached (60% reduction in metaphases compared to control). In the presence of 1.8% (v/v) S9-fraction, Ketjenflex[®] 8 was tested up to 1400 μ g/ml for 3 hours exposure with 24 hours fixation time. Appropriate toxicity was reached at this dose level (49% reduction in metaphases compared to control).

In the second cytogenetic assay, Ketjenflex[®] 8 was tested up to 500 μ g/ml for 24 and 48 hours continuous exposure with 24 and 48 hours fixation time in the absence of S9-mix. Appropriate toxicity was reached at this dose level (59 % (24 hours) and 51% (48 hours)

reduction in metaphases compared to control). In the presence of S9-mix, Ketjenflex® 8 was tested up to 1600 µg/ml for 3 hours exposure with 48 hours fixation time. Ketjenflex® 8 precipitated in the culture medium at this dose level. The recommended toxicity was not reached in this experiment.

In the first cytogenetic assay in the absence of S9-mix, and in the second cytogenetic assay both in the absence and presence of S9-mix, Ketjenflex® 8 did not induce a statistically significant or biologically relevant increase in the number of cells with chromosome aberrations.

In the first cytogenetic assay in the presence of S9-mix, no increase in the number of cells with chromosome aberrations was observed at concentration levels up to and including 1200 µg/ml. Only at the highest tested cytotoxic concentration of 1400 µg/ml, a statistically significant increase in number of cells with chromosome aberrations was observed, both when gaps were included and excluded.

It was noted that Ketjenflex® 8 increased the number of polyploidy cells in the absence of S9-mix in the first cytogenetic assay. This may indicate that Ketjenflex® 8 has the potential to disturb mitotic processes and cell cycle progression.

Conclusions from study authors

It was concluded that the test was valid, and that Ketjenflex® 8 is not clastogenic in human peripheral lymphocytes in the absence of S9-mix. Ketjenflex® 8 was found to be clastogenic in human peripheral lymphocytes in the presence of S9-mix when tested up to the recommended toxicity. It was noted that there was a high occurrence of exchanges. In addition, Ketjenflex® 8 increased the number of polyploid cells in the absence of S9-mix, which indicate that Ketjenflex® 8 may also have aneugenic potential.

Comments from VKM

VKM agrees with the study authors that Ketjenflex® 8 was clastogenic in human peripheral lymphocytes in the presence of S9-mix. However, it is noted that the S9-concentration of 1.8% used in this study may not be optimal since it was shown in the L5178Y mouse lymphoma assay (7) that the optimal S9-concentration was 12%, and that 8% S9 did not induce mutants.

3.3.5.2 *In vivo*

Rat bone marrow micronucleus test

Micronucleus test in bone marrow cells of the rat with Ketjenflex® 8 (NETSA) (2006). NOTOX Project 452824 (9)

Guidelines: The OECD Guideline for the Testing of Chemicals, No. 474: Mammalian Erythrocyte Micronucleus Test (adopted July 21, 1997) (14), and Commission Directive 2000/32/EC of 19 May 2000, Annex 4C, B.12. Mutagenicity – *In vivo* mammalian erythrocyte micronucleus test (21), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)

Species/Strain: Rat, Wistar WI (SPF), both sexes, six weeks old

Group size:	5 male and 5 female rats per sampling time in each treatment group
Test substance:	Ketjenflex [®] 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Dose levels:	100.0, 200.0, 400.0 mg/kg bw/day
Route:	Oral, a single intubation
Vehicle:	Propylene glycol
Sacrifice times:	24 hours and 48 hours (highest dose only)
GLP:	In compliance

Ketjenflex[®] 8 was tested for its clastogenic/aneugenic potential in bone marrow cells of rats. Dose selection was based on findings in the pre-experiments for toxicity with 1 male and 1 female in three dose groups (2000.0, 1000.0 and 500.0 mg/kg bw) and 3 males and 3 females dosed with 400.0 mg/kg bw. Based on this pre-test, 400.0 mg/kg bw was selected as maximum tolerated dose and used as the highest dose in the main experiment (without explanation). Bone marrow was sampled from the femurs of the rats treated with Ketjenflex[®] 8, 24 or 48 hours (highest dose only) after dosing. Bone marrows from the negative and positive control groups were harvested 24 and 48 hours after dosing, respectively. Slides were prepared from the bone marrow preparations, stained and evaluated blindly for the number of polychromatic erythrocytes (PCE) with micronuclei. Two thousand PCE per animal were analysed. In addition, the ratio between polychromatic to normochromatic erythrocytes was determined for each animal. Positive (cyclophosphamide) and negative (vehicle; propylene glycol) controls were included.

Results

All rats given Ketjenflex[®] 8 were lethargic after dosing. At the 400.0 mg/kg bw dose levels, 8 males and 7 females also showed ventral recumbency. The other 2 males and 3 females showed ataxia after receiving this dose. All rats of the dose level of 200.0 and 100.0 mg/kg bw also showed ataxia after dosing, except one male rat of the dose level 200.0 mg/kg bw which showed central recumbency. All rats recovered within 18 hours after treatment. The animals in the control groups showed no abnormalities after dosing.

No increase in the mean frequency of micronucleated polychromatic erythrocytes was observed in the polychromatic erythrocytes of the bone marrow of rats treated with Ketjenflex[®] 8 compared to the vehicle-treated rats.

The rats that were treated with Ketjenflex[®] 8 showed no decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the negative (vehicle) controls, which reflects a lack of toxic effects of this product on the erythropoiesis.

The incidence of micronucleated polychromatic erythrocytes in the bone marrow of all negative control rats was within the historical solvent control data range. Cyclophosphamide, the positive control substance, induced a statistically significant increase in the number of micronucleated polychromatic erythrocytes in both sexes.

Conclusions from study authors

It was concluded that Ketjenflex[®] 8 was not clastogenic or aneugenic in the *in vivo* micronucleus test under the experimental conditions described.

Comments from VKM

Although toxicity was not observed at the target organ (bone marrow), the toxic effects on CNS, i.e. lethargy etc., observed in all dose groups, indicate systemic effect of the test substance. There was a high occurrence of exchanges in the *in vitro* chromosomal aberration assay with peripheral human lymphocytes (8). However, clastogenic effects such as exchanges cannot be excluded by this *in vivo* micronucleus assay, which can detect only breaks and lagging chromosomes.

Micronucleus evaluation in bone marrow from some animals from the repeated dose 90-day oral toxicity study with Ketjenflex® 8 (NETSA) by daily gavage in the rat (2006). NOTOX Project 441282 (10)

Guidelines:	The OECD Guideline for the Testing of Chemicals, No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents (adopted 21 September 1998) (14), and Commission Directive 2001/59/EC of 6 August 2001, Annex 5D, B.26. Subchronic oral toxicity test, repeated dose 90-day oral toxicity study in rodents (19), amending Council Directive 67/548/EEC of 27 June 1967, Annex V (18)
Species/Strain:	Rat, Wistar Crl(WI) BR (outbred, SPF-quality), both sexes, six weeks old
Group size:	5 male and 5 female rats per dose group
Test substance:	Ketjenflex® 8 (CAS no. 8047-99-2)
Batch:	504656008
Purity:	Minimum 99.6%
Dose levels:	0 (vehicle) and 400.0 mg/kg bw/day
Route:	Oral, gavage once daily for at least 90 days, 7 days/week
Vehicle:	Propylene glycol
GLP:	In compliance

Results

Five rats of each sex treated with vehicle (propylene glycol) and the highest dose of Ketjenflex® 8, 400.0 mg/kg bw/day, from the 90-day oral toxicity study described above (5) were examined for micronuclei in the bone marrow taken from the femur at termination.

No increase in the mean frequency of micronucleated polychromatic erythrocytes was observed in the polychromatic erythrocytes of the bone marrow of rats treated with 400.0 mg/kg bw Ketjenflex® 8 compared to the vehicle-treated rats.

The rats that were treated with 400.0 mg/kg bw/day Ketjenflex® 8 showed no decrease in the ratio of polychromatic to normochromatic erythrocytes compared to the negative (vehicle) controls, which reflects a lack of toxic effects of this product on the erythropoiesis.

Conclusions from study authors

It was concluded that Ketjenflex® 8 was not clastogenic or aneugenic in the *in vivo* micronucleus test under the experimental conditions described.

Comments from VKM

Although toxicity was not observed at the target organ (bone marrow), the toxic effects on CNS, i.e. lethargy etc., observed in all dose groups, indicate systemic effect of the test

substance. There was a high occurrence of exchanges in the *in vitro* chromosomal aberration assay with peripheral human lymphocytes (8). However, clastogenic effects such as exchanges cannot be excluded by this *in vivo* micronucleus assay, which can detect only breaks and lagging chromosomes.

General comments from VKM on mutagenicity/genotoxicity

NETSA was tested for mutagenic potential in two non-guideline bacterial reverse mutation tests. In the first study, NETSA was mutagenic in TA1535 without S9-mix. The second study was inconclusive. NETSA was genotoxic (presumably clastogenic) in the presence of S9-mix in the mouse lymphoma assay. NETSA was clastogenic in human lymphocytes *in vitro* in the presence of S9-mix, and induced a high occurrence of exchanges. There was also an indication that the test product has an aneugenic potential. The clastogenic/aneugenic potential, i.e. the ability to cause structural and numerical chromosomal aberrations, was not confirmed in two *in vivo* micronucleus assays. However, exchanges will not be detected in the micronucleus assay.

3.3.6 Carcinogenicity

No data found.

3.3.7 Reproductive toxicity

No data found.

3.4 Summary of hazards

3.4.1 General toxicity

In the three available repeated dose toxicity studies of NETSA in rats, early, transient effects on the central nervous system (CNS), such as lethargy and uncoordinated movements, were observed. The overall LOAEL value for these acute effects on CNS was 18.75 mg/kg bw/day. It should be noted that this value is of similar magnitude as the subchronic LOAEL value. Increased absolute and relative weights of liver and kidneys were observed in a 90-day study, indicating liver and kidney toxicity. The LOAEL value for these subchronic effects was 25 mg/kg bw/day based on significantly increased relative liver weight in female rats.

3.4.2 Mutagenicity/genotoxicity

NETSA was tested for mutagenic potential in two non-guideline bacterial reverse mutation assays. In the first study, NETSA was a mutagen in TA1535 without S9-mix. The second study was inconclusive. NETSA was genotoxic (presumably clastogenic) in the presence of S9-mix in the mouse lymphoma assay. NETSA was clastogenic in human lymphocytes *in vitro* in the presence of S9-mix, and induced a high occurrence of exchanges. There was also an indication that the test product has an aneugenic potential. The clastogenic and aneugenic potential, i.e. the ability to cause structural and numerical chromosomal aberrations, was not confirmed in two *in vivo* micronucleus assays. However, exchanges will not be detected in the micronucleus assay. Therefore, to be able to determine with certainty the mutagenic and genotoxic potential of NETSA, the following studies should be performed:

- A bacterial reverse mutation assay *in vitro* (Ames test) performed according to the OECD guideline no. 471 (14).
- A chromosomal aberration assay on rat bone marrow *in vivo* according to the OECD guideline no. 475 (14).
- A Comet assay *in vivo* on rat liver and kidney according to proposed guideline from 2007 (24).

Genotoxicity analysis using a quantitative structure-activity relationship (QSAR) prediction program (Multicase, version 2007) gave positive results for *N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) in Direct Ames sub-model without S9-mix and in Ames sub-model for potency. The *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7) was positive only in Ames sub-model for potency. In a cancer model (Multicase, FDA), the ortho (2-) isomer was positive, whereas the para (4-) isomer was negative. The results of Ames tests from NTP also showed that the mixture of ortho and para isomers (CAS no. 8047-99-2) was weakly positive, whereas the para isomer alone (CAS no. 80-39-7) was negative. A methyl group placed in ortho position therefore seems to have a significant effect on toxicity of these compounds.

Some of the data submitted for evaluation are from studies using mixtures of ortho and para isomers of NETSA in unknown percentages. Other available data, as mentioned above, indicate that the ortho isomer has greater potential for mutagenicity and genotoxicity than the para isomer of NETSA. Therefore, in new studies, the “worst case” mixture of NETSA should be used, i.e. with the highest concentrations of the ortho isomer. If the meta isomer is used in connection with food packaging materials, this isomer should also be tested. The specification/composition of the mixture should be described in detail.

3.5 Exposure characterization

The basis for our previous risk assessment of NETSA in 2004 (1) was detection of migration of NETSA from printed multilayer cheese films collected on the Norwegian market in 2003 to food simulants (water and olive oil) (25). In that study, the highest values of NETSA found to migrate were 13 mg/kg water simulant and 4.1 mg/kg olive oil simulant. According to a national food intake study of adults in Norway (NORKOST, $n=2672$) (26), the mean intake is 34 g cheese/day, and the high intake (95 percentile, consumers only) is 84 g/day. From intake of cheese, the intake of NETSA will be 0.4 and 1.1 mg/day, or 6.7 and 18.3 $\mu\text{g}/\text{kg}$ bw/day, for mean and high intake, respectively, based on the migration value of 13 mg NETSA per kg water simulant and 60 kg bw. Based on the migration value of 4.1 mg NETSA per kg olive oil simulant and the intake of cheese from the study of adults in Norway, the intake of NETSA will be 0.1 and 0.3 mg/day, or 1.7 and 5.0 $\mu\text{g}/\text{kg}$ bw/day, for mean and high intake, respectively, from cheese wrapped in these films. It should be noted that additional exposure could occur from other foods contaminated by printing ink from packaging materials.

The use of plasticizers in ink is not yet regulated within the EU. The applicant has provided new experimental data for determining if migration up to 5 mg/kg food or food simulant can be allowed according to the European Food Safety Authority (EFSA)’s Note for Guidance for Food Contact Materials (15). Using the EU model for exposure from food contact materials, i.e. assuming a daily intake of 1 kg of food containing NETSA by a person weighing 60 kg, a migration of 5 mg/kg would result in an exposure to NETSA of 0.08 mg/kg bw/day. From a

migration of 13 mg/kg food simulant as detected in Norway from cheese films (25), the resulting exposure would be 0.22 mg/kg bw/day.

3.6 Risk characterization

Because the potential mutagenic and genotoxic properties of NETSA was not resolved, it was not possible to perform a complete risk characterization based on the earlier and newly submitted data, and a TDI or TWI value could not be established.

In addition, the submitted data on general toxicity did not allow establishment of a NOAEL for chronic toxicity. The LOAEL obtained for acute effects on CNS was 18.75 mg/kg bw/day and the LOAEL in subchronic studies for effects on liver weight was 25 mg/kg bw/day. In this case, for a derivation of a TDI or an acceptable margin of safety (MOS), an uncertainty factor additional to the default uncertainty factor of 100 would be needed. Hence, in spite of possible negative mutagenicity data, a migration value of 13 mg/kg food simulant as measured in Norway from cheese films, or even a migration limit of 5 mg/kg food as suggested by the applicant, and using the EU exposure model, are incompatible with the submitted data on acute and subchronic toxicity.

4 CONCLUSIONS

The general toxicity data indicate that NETSA induces acute transient effects on CNS in rats, such as lethargy and uncoordinated movements. Increased absolute and relative weights of liver and kidneys in rats were observed in a 90-day rat study, indicating liver and kidney toxicity.

The mutagenic potential of NETSA in bacterial reverse mutation assay *in vitro* is still inconclusive. NETSA showed clastogenic/aneugenic potential in the presence of activation *in vitro*, but this effect is still inconclusive *in vivo*. Further studies are therefore needed to clarify the mutagenic and genotoxic potential of NETSA, and it is therefore not possible to establish a TDI or TWI value for NETSA based on the new submitted data and earlier available data. Hence, a conclusion cannot be reached regarding maximum allowable migration levels of NETSA, as requested by the applicant, because the mutagenic and genotoxic potential of NETSA was not determined with certainty.

The Panel noted that in spite of possible negative mutagenicity data, when using the EU exposure model a migration value of 13 mg/kg food simulant as measured in Norway from cheese films, or even of a migration limit of 5 mg/kg food as suggested by the applicant, were found to be incompatible with the submitted data showing acute and subchronic toxicity with LOAELs in the range of 18.75 to 25 mg/kg bw/day.

5 APPENDIX

***N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1)**

In a research paper on quantitative structure-toxicity relationship models for assessing dermal sensitization using the guinea pig maximization test results (27), *N*-ethyl-2-toluenesulfonamide (CAS no. 1077-56-1) was listed as a non-sensitizer in a table, with reference H.I. Maibach, personal communication.

***N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7)**

Ames/Salmonella mutagenicity assay performed by The National Toxicology Program (NTP) in U.S.A. (20)

Guideline:	Standard NTP protocol
Species/Strain:	<i>Salmonella typhimurium</i> , TA97, TA98, TA100, TA1535 and TA 1537
Replicates:	One assay. Replicates not stated
Assay conditions:	Preincubation assay, both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and male Syrian hamster liver S9
Test substance:	<i>N</i> -ethyl-4-toluenesulfonamide (CAS no. 80-39-7)
Batch:	Not stated
Purity:	Not stated
Concentrations:	33.0, 100.0, 333.0, 1000.0, 1666.0 (-S9, not in TA 1537), 3333.0, 6666.0 (+S9) µg/plate
Solvent:	DMSO
GLP:	Not stated

In 1986, *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7) was evaluated by NTP. The compound was tested in TA97, TA98, TA100, TA1535 and TA1537, both with and without S9-mix (10% rat liver, 30% rat liver, 10% hamster liver and 30% hamster liver). Raw data was not shown, only the mean and SD.

Results

N-ethyl-4-toluenesulfonamide (CAS no. 80-39-7) was negative in all tester strains both with and without S9-mix.

Conclusions from study authors

The overall results were that the *N*-ethyl-4-toluenesulfonamide (CAS no. 80-39-7) was not mutagenic in bacteria.

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