EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

2-Hydroxyethylamino-5-nitroanisole

COLIPA n° B52

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 3rd plenary of 8 July 2009

About the Scientific Committees
Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS
The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

Scientific Committee members
Jürgen Angerer, Ulrike Bernauer, Claire Chambers, Mohammad Chaudhry, Gisela Degen, Gerhard Eisenbrand, Corrado Galli, Thomas Platzek, Suresh Chandra Rastogi, Vera Rogiers, Christophe Rousselle, Tore Sanner, Kai Savolainen, Jacqueline Van Engelen, Maria Vinardell, Rosemary Waring, Ian R. White

Contact
European Commission
Health & Consumers
Directorate C: Public Health and Risk Assessment
Unit C7 - Risk Assessment
Office: B232 B-1049 Brussels
Sanco-Sc6-Secretariat@ec.europa.eu

© European Commission 2009
(ISSN)

The opinions of the Scientific Committees present the views of the independent scientists who are members of the committees. They do not necessarily reflect the views of the European Commission. The opinions are published by the European Commission in their original language only.

http://ec.europa.eu/health/ph_risk/risk_en.htm

ACKNOWLEDGMENTS

Prof. J. Angerer
Dr. C. Chambers
Dr. M. Chaudhry
Prof. G. Eisenbrand
Prof. T. Platzek (chairman)
Dr. S.C. Rastogi
Dr. C. Rousselle
Prof. T. Sanner
Prof. M. Vinardell
Prof. R. Waring
Dr. I.R. White

External experts

Dr. M.-L. Binderup National Food Institute, Denmark (rapporteur)

Keywords: SCCS, scientific opinion, hair dye, B52, 2-hydroxyethylamino-5-nitroanisole, directive 76/768/ECC, CAS 66095-81-6, EINECS 266-138-0

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Opinion on 2-hydroxyethylamino-5-nitroanisole, 8 July 2009

TABLE OF CONTENTS

1. BACKGROUND

According to COLIPA[1], submission I for 2-Hydroxyethylamino-5-nitroanisole was sent in May 1995.

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted during its plenary meeting of 20 May 1998 an opinion (XXIV/1289/97) with the conclusion that:

"The acute oral toxicity of 1-methoxy-2-(ß-hydroxyethyl)-amino-5-nitrobenzene in the rat is estimated to be >2000 mg/kg bw for females and in the region of 2000 mg/kg bw for males. The substance can be classified as slightly irritating to the eyes and not irritating to the skin. Percutaneous absorption of a formulation was 0.64% in absence and 0.29% in presence of hair. In a 28 day study in rats, 100 mg/kg/day was the NOAEL. In a teratogenicity study, no signs of maternal or foetal toxicity were observed after administration of 250 mg/kg. The substance was found to be not mutagenic."

Submission II was sent in July 2005 by COLIPA. According to this submission, 2-Hydroxyethylamino-5-nitroanisole is used in semi permanent hair formulations at a maximum concentration of 0.2%.

The Scientific Committee on Cosmetic Products (SCCP) adopted at its 13th plenary meeting on 2 October 2007 an opinion (SCCP/1099/07) with the conclusion that "*This risk assessment relates to the use of 2-hydroxyethylamino-5-nitroanisole in non-oxidative hair dye formulations only.*
Since 2-hydroxyethylamino-5-nitroanisole was positive in a gene mutation tests in bacteria, a proper genotoxicity tests covering in vivo gene mutations is essential to definitively conclude on the genotoxicity of 2-hydroxyethylamino-5-nitroanisole.
2-Hydroxyethylamino-5-nitroanisole is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb."

Submission III was sent in April 2009, enclosing an *in vivo* Unscheduled DNA Synthesis (UDS) assay.

2-Hydroxyethylamino-5-nitroanisole (CAS No 66095-81-6) and its salts are currently regulated in Annex III, part 2 under entry 29 for use in non-oxidising colouring agents for hair dyeing in a concentration up to 1.0%.

2. TERMS OF REFERENCE

1. *Does SCCS consider 2-Hydroxyethylamino-5-nitroanisole safe for use in non-oxidative hair dyes with a concentration of maximum 0.2 % taken into account the scientific data provided?*

2. *And/or does the SCCS has any further scientific concerns with regard to the use of 2-Hydroxyethylamino-5-nitroanisole in non-oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

3. OPINION

3.1. Chemical and Physical Specifications

Taken from SCCP/1099/07

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2-Hydroxyethylamino-5-nitroanisole (INCI)

3.1.1.2. Chemical names

1-methoxy-2-(β-hydroxyethyl) amino-5-nitro-benzene
Ethanol, 2-[(2-methoxy-4-nitrophenyl)amino]- (CAS name)

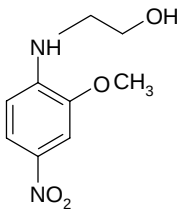
3.1.1.3. Trade names and abbreviations

IMEXINE FM

3.1.1.4. CAS / EINECS number

CAS: 66095-81-6
EINECS: 266-138-0

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_9H_{12}N_2O_4$

3.1.2. Physical form

Orange-red powder, almost odourless

3.1.3. Molecular weight

Molecular weight: 212.21

3.1.4. Purity, composition and substance codes

Batch 0507391- data reported for:
Chemical identification by IR and UV
Chemical characterization by NMR, Mass spectrometry and elemental analysis

Titre: > 98.5 g/100g (Determination by spectrophotometry)
Purity by HPLC: Relative purity > 99%

Impurities
- 2-methoxy-4-nitro-phenylamine: < 0.2 g/100g
- 4-Nitro-2-methoxyphenyl-N-(β-chloroethylcarbamate): < 0.1 g/100g
- 3-(2-Methoxy-4-nitro-phenyl)-oxazolidin-2-one: < 0.1 g/100g

Ash content: < 1 g/100g
Heavy Metals: < 10 µg/g

- As, Sb, Hg: each < 5 mg/kg
- Cd: < 10 mg/kg
- Pb: < 20 mg/kg

Comparison of Batches

	0507391	**Op. T39**	**Op. T37**
Appearance	Orange-red powder		
Titre by spectrophotometry (g/100g)	99.0	99.9	> 99
Water content (g/100g)	0.06	0.06	0.12
Melting point (°C)	84 [1]	83 [1]	86.5-89.5 [2]
HPLC Profile **UV purity (%)[3]**	99.5	99.5	Conforms to the standard
Impurities HPLC (g/100g) **2-methoxy-4-nitrophenylamine** **4-nitro-2-methoxyphenyl-N-(β-chloroethylcarbamate)** **3-(2-methoxy-4-nitrophenyl)-oxazolidin-2-one**	 D < 0.1 D < 0.1 ND < 0.1	 0.09 D < 0.1 ND < 0.1	 0.12 0.02 D < 0.01
Residual solvent GC (µg/g) **Isopropanol**	 200	 400	 580
UV spectrum	The UV spectra are comparable		
IR spectrum	In accordance with the proposed structure		
Mass spectrum	Compatible with the proposed structure		
1H and 13C NMR spectra	In accordance with the proposed structure		

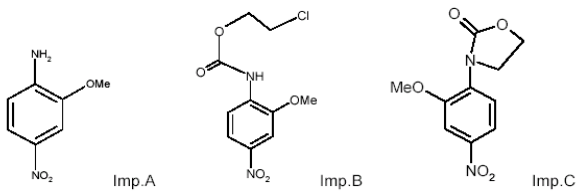
[1] DSC (differential scanning calorimetry)
[2] Thermomicroscopic method
[3] UV detection/ UV purity – Area% without response factor
Irrespective of residual solvents, salts and other non detectable products
D detected
ND not detected

Each impurity content was carried out against a reference standard considered as pure.

3.1.5. Impurities / accompanying contaminants

Possible impurities may originate from:

- Reagents and intermediate reaction products:
- 2-methoxy-4-nitro-phenylamine (A)
- 4-nitro-2-methoxyphenyl-N(β-chloroethylcarbamate) (B)
- 3-(2-methoxy-4-nitro-phenyl)-oxazolidin-2-one (C)



- Impurity A was detected:
  - < 0.1 g/100g for batches 0507391 and Op. T39
  - 0.12 g/100g for batch Op. 37

- Impurity B was detected:
  - < 0.1 g/100g for batches 0507391 and Op. T39
  - 0.02 g/100g for batch Op. 37

- Impurity C content was below 0.1g/100g
  - Not detected for batches 0507391 and Op. T39
  - Detected for batch Op.37

Each impurity content was carried out against a reference standard considered as pure.

- Residual solvents
- Isopropanol

3.1.6. Solubility

Water solubility: 603 ± 5 mg/l at 20°C ± 0.5 °C (according to EEC method A6)

Solubility (g/100ml - 22°C after 24 h)
- Ethanol: $1 \leq S < 10$
- DMSO: $S \geq 20$

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 1.52 at 25°C ± 1°C at pH 7.11 (EEC method A8 (HPLC method))

3.1.8. Additional physical and chemical specifications

Melting point:	83°C to 90°C (according to analytical method)
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH:	/
UV_Vis spectrum	λ_{max} 228.0, 264.0 and 400.0 nm (in 95% ethanol)

3.1.9. Homogeneity and Stability

The homogeneity of the test item at 4 and 200 mg/ml in 0.5% MC on the day of preparation was satisfactory (average variability 1% for both concentrations).
The stability of the test item in dosage forms over a 4-hour period at room temperature, protected from light and under inert gas atmosphere was tested in the following dosage forms with the respective results indicated in brackets:

- 0.1 mg/ml in DMSO (Deviation in hour 4 from initial value in hour 0: 9%)
- 250 mg/ml in DMSO (Deviation in hour 4 from initial value in hour 0: 9%)
- 5 mg/ml in acetone/olive oil (Deviation in hour 4 from initial value in hour 0: -4%)
- 100 mg/ml in acetone/olive oil (Deviation in hour 4 from initial value in hour 0: 6%)

Ref.: 12

General Comments to physico-chemical characterisation

* 2-Hydroxyethylamino-5-nitroanisole is a secondary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
* No documentation was provided to support the data presented in the table "comparison of batches"
* No documentation was submitted on the stability of the test substance in marketed products.

3.2. Function and uses

2-Hydroxyethylamino-5-nitroanisole is used in semi-permanent hair dye formulations at a maximum concentration of 0.2%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCP/1099/07

Guideline:	OECD 420, "fixed dose method"
Species/strain:	rat, Sprague-Dawley Rj: SD (IOPS Han)
Group size:	5 female rats
Test substance:	2-hydroxyethylamino-5-nitroanisole suspended in 0.5% methylcellulose
Batch:	0507391
Purity:	99.0%
Dose:	500 mg/kg bw
Route:	oral, gavage
GLP:	in compliance

In an initial test, the test substance was administered at 1000 mg/kg bw. As mortality occurred at this dose, the dose of 500 mg/kg bw was chosen next. Since no mortality occurred at this dose, the test substance was administered to 4 additional animals. In the observation period of 2 weeks clinical signs and mortality were recorded and body weights were recorded on day 1, 8, and 15. All animals were sacrificed on day 15 and subjected to macroscopic examination.

Results
No mortality was observed at 500 mg/kg bw. Hypoactivity or sedation, pilorection, dyspnea and yellow extremities were noted on day 1 in all animals. The overall body weight was not affected and macroscopic examination revealed no abnormalities.

Conclusion
The maximal non-lethal dose of the test substance was 500 mg/kg bw and the minimal lethal dose is 1000 mg/kg bw under the experimental conditions.

Ref.: 1

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2. Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCP/1099/07

Guideline:	OECD 404
Species:	New Zealand White
Group:	3 male
Substance:	2-hydroxyethylamino-5-nitroanisole
Batch:	0507391

Purity:	99%
Dose:	0.5 ml of 10%, 3 min and 1 h in one animal, 4 hour semi occlusion in 3 animals
Vehicle:	aqueous suspension of 0.5% methylcellulose
GLP:	in compliance

The acute skin irritation potential of 2-hydroxyethylamino-5-nitroanisole was evaluated following a single dermal application to rabbits An aliquot of a 10% dilution of 2-hydroxyethylamino-5-nitroanisole was applied to the dermal application site (dorsal flank clipped free of hair) on a dry gauze pad and covered with a semi-occlusive dressing. The gauze pad and semi-occlusive dressing were held in place for up to four hours by a restraining bandage. Following bandage removal, the dressing was removed and remaining test item wiped off by means of a moistened cotton pad. The application site was assessed for cutaneous reactions.

After a 3-minute exposure (one animal), a very slight erythema was noted on days 1 and 2. A yellow coloration of the skin was also noted from day 1 up to day 4. After a 1-hour exposure (one animal), a yellow coloration of the skin, which could have masked a well-defined erythema on day 1 or a very slight erythema on days 2 and 3, was noted from day 1 up to day 4. After a 4-hour exposure (three animals), a very slight erythema was noted in 1/3 animals on days 1 and 2. A yellow coloration of the skin was noted in all animals from day 1 up to day 4; this coloration could have masked a very slight erythema in 1/3 animals from day 1 up to day 3. No oedema was observed following the 3-minute, 1 and 4-hour exposures.

Under the conditions of the study, a 10% dilution of 2-hydroxyethylamino-5-nitroanisole showed transient, slight irritant potential to rabbit skin.

Ref.: 2

3.3.2.2. Mucous membrane irritation

Taken from SCCP/1099/07

Guideline:	OECD 405
Species:	New Zealand White
Group:	3 male
Substance:	2-hydroxyethylamino-5-nitroanisole
Batch:	0507391
Purity:	99%
Dose:	0.1ml of 1%, in left conjuctival sac
Vehicle:	aqueous suspension of 0.5% methylcellulose
GLP:	in compliance

The acute ocular irritation potential of 2-hydroxyethylamino-5-nitroanisole diluted at 1% was evaluated following instillation into the left conjunctival sac of 3 rabbit eyes. The opposite, untreated eye served as a control. Observations were made at 1, 24, 48 and 72 hours. There were no ocular reactions observed in any of the animals tested.

Under the conditions of the study, 1% 2-hydroxyethylamino-5-nitroanisole was non-irritating to rabbit eyes.

Ref.: 3

3.3.3. Skin sensitisation

Taken from SCCP/1099/07

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	CBA/J female mice
Group:	28 animals, (5 groups of 4 treated, negative, positive controls)
Substance:	2-hydroxyethylamino-5-nitroanisole
Batch:	0507391
Purity:	99%
Dose:	0.5, 1, 2.5, 5 and 10%
Vehicle:	acetone/olive oil (4:1)
Control:	negative: vehicle; positive α hexylcinnamaldehyde 25% in acetone/olive oil
GLP:	in compliance

The skin sensitising potential of 2-hydroxyethylamino-5-nitroanisole was evaluated in a Local Lymph Node Assay (LLNA) in mice. The assay was performed up to the maximal practicable concentration (10% in acetone/olive oil (4:/1; v/v)).

Except for a colouration of the ears noted at concentrations of 1% and higher, no cutaneous reactions and no increases in ear thickness were observed in the treated groups.

Dilution %	Stimulation Index
0.5	1.36
1	0.89
2.5	1.58
5	0.80
10	1.31
α-hexylcinnamaldehyde 25%	6.13

No positive lymphoproliferative responses were observed in the treated groups.

Conclusion
On the basis of the results of this murine LLNA, 2-hydroxyethylamino-5-nitroanisole at a maximal dilution of 10% was considered to have no skin sensitising potential.

Ref.: 4

3.3.4. Dermal / percutaneous absorption

Taken from SCCP/1099/07

Guideline:	OECD 428
Tissue:	human skin; dermatomed thickness 400µm
Group size:	total of 7 intact membranes, 3 female donors
Diffusion cells:	flow through
Skin integrity:	tritiated water. $K_p < 2.5 \times 10^{-3}$ cm h^{-1} selected
Test substance:	2-hydroxyethylamino-5-nitroanisole [^{14}C]- 2-hydroxyethylamino-5-nitroanisole (labelled)
Batch:	0507391 3497.141 (labelled)
Purity:	99% (w/w) 98.55% (labelled)
Test item:	0.21% 2-hydroxyethylamino-5-nitroanisole in semi permanent dye formulation

Doses:	12.98 mg formulation (42.6µg cm^2 test substance)
Receptor fluid:	PBS with 0.01% sodium azide
Solubility receptor fluid:	< 1mg mL^{-1}
Stability:	stable
Method of Analysis:	liquid scintillation counter
GLP:	compliant

The *in vitro* percutaneous absorption of [14C]- 2-hydroxyethylamino-5-nitroanisole from a typical semi-permanent hair colouring formulation was determined in human dermatomed skin mounted on diffusion cells, using phosphate-buffered saline containing 0.01% sodium azide (w/v) as the receptor fluid.

About 20 mg/cm^2 of the formulation containing radiolabelled 2-hydroxyethylamino-5-nitroanisole was applied to the skin surface. To mimic actual use conditions, 2-hydroxyethylamino-5-nitroanisole was incorporated into a typical hair colouring formulation at 0.21% (w/w). The test formulation was in contact with skin for 30 minutes. After this time, the remaining formulation on the skin surface was removed using a standardized washing procedure, simulating use conditions. Twenty-four hours after application, the percutaneous absorption of 2-hydroxyethylamino-5-nitroanisole was estimated by measuring its concentration in the following compartments (liquid scintillation counting): skin excess, stratum corneum (isolated by tape strippings), skin (living epidermis plus dermis) and receptor fluid.

	% of dose applied	**µg $_{eq}$ cm^{-2}**
Skin wash	93.4 ± 3.4	39.8 ± 1.6
Dislodgeable dose	93.5 ± 3.3	39.8 ± 1.5
Stratum corneum	0.17 ± 0.05	0.07 ± 0.02
Skin (epidermis + dermis)	0.05 ± 0.03	0.02 ± 0.01
Receptor fluid	1.43 ± 0.67	0.61 ± 0.29
Unabsorbed dose	93.7 ± 3.3	39.9 ± 1.5
Absorbed dose	**1.48 ± 0.69**	**0.63 ± 0.30**
Total recovery	95.2 ± 2.9	40.5 ± 1.3

The amounts considered as absorbed (sum of the amounts measured in the living epidermis, dermis, and receptor fluid) represented 0.63 ± 0.30 µg $_{eq}$ cm^{-2} (1.48 **±**0.69% of the applied dose); range 0.36 – 1.05 µg $_{eq}$ cm^{-2} (0.83 **–** 2.50% of the applied dose).

As only a total of 7 chambers were used from a total of 3 donors, the dermal absorption of 2-hydroxyethylamino-5-nitroanisole incorporated at 0.21% in a typical semi-permanent hair colouring formulation should be the maximum observed for use in MOS calculation: A_{max} 1.05 µg $_{eq}$ cm^{-2} (2.50% of the applied dose).

Ref 11

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

Taken from SCCP/1099/07

Guideline:	OECD 408
Species/strain:	rats, Sprague-Dawley Crl:CD(SD)BR strain (VAF plus)
Group size:	10 animals per sex and dose group
Test substance:	Imexine FM suspended in 0.5 % carboxymethylcellulose

Batch:	OpT 39
Purity:	99.9%
Dose:	0, 25, 100 and 500 mg/kg bw/day
Route:	oral, gavage
Exposure:	13 weeks
GLP:	in compliance

The test substance was given orally by gavage at dose levels 25, 100 or 500 mg/kg bw/day while the controls received the vehicle 0.5% carboxymethylcellulose alone. The dose levels were selected based on a 4-week range finding study (NOAEL 100 mg/kg bw/day). Animals were observed daily for mortality and clinical signs. Body weights and feed consumption were recorded weekly. Ophthalmoscopy was performed with all animals before the start of treatment and on control and high dose animals during week 13. Haematology, blood chemistry and urinalysis were investigated during week 12/13. At study end necroscopy was performed and several tissues of control and high dose animals were examined microscopically.

Results
No substance-related deaths and no clinical signs were observed but yellow discolouration of the skin, mammary gland and urine was observed. The latter effect prevented the evaluation of most urine parameters in the high dose group. Body weight and feed consumption were not affected and there were no ocular abnormalities found. Relative spleen and liver weights of high dose females were significantly increased. In haematology elevated prothrombin times (males and females) and fibrinogen levels (males) were observed in the high dose. Also in the high dose changes in the mean blood urea nitrogen (females) and mean alanine aminotransferase levels (males) were recorded as well an increase in urinary volume (males and females). Macroscopic and microscopic examinations of tissues revealed no pathological evidence of toxicity.

Conclusion
The NOAEL of Imexine FM in this subchronic oral toxicity study in rats is 100 mg/kg bw/day.
Ref.: 5

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Taken from SCCP/1099/07

Bacterial gene mutation assay

Guideline:	/
Strain:	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrA.
Replicates:	3 replicates in 2 individual experiments both in the presence and absence of S9-mix.
Test substance:	Imexine FM
Solvent:	DMSO
Batch:	Op T 39
Purity:	/
Concentrations:	experiment 1: 8 - 5000 µg/plate without and with S9-mix experiment 2: 250 - 4000 µg/plate without and with S9-mix

Treatment:	direct plate incorporation with approximately 65 h incubation time without and with S9-mix
GLP:	in compliance

Imexine FM was investigated for the induction of gene mutations in *Salmonella typhimurium* and *Escherichia coli* (Ames test). Test concentrations were based on the results in a toxicity range finder test with strains TA98 and WP2 uvrA. Toxicity was evaluated on the basis of the appearance of the bacterial lawn. Imexine FM was tested up to the prescribed maximum concentration of 5000 µg/plate using the direct plate incorporation method. Liver S9 fraction from β-naphthoflavone and sodium phenobarbitone-induced rats was used as exogenous metabolic activation system. Justified negative and positive controls were concurrently tested.

Results
Toxicity, reported as a reduced background lawn, was only seen in experiment 1 in TA1535, TA1537 and TA100 (without S9-mix only).
In experiment 1 both without and with S9-mix a biologically relevant, dose dependent increase in the number of revertants was seen in the TA98 strain. In experiment 2, a biologically relevant, dose dependent increase was seen in TA98, TA100 (without S9-mix only) and TA 1537.

Conclusion
Under the experimental conditions used Imexine FM was genotoxic (mutagenic) in the gene mutation tests in bacteria both in the absence and the presence of S9 metabolic activation.
Ref.: 6

Comment
Although the study is from 1994 it was not performed according to OECD guideline 471. However, negative and positive controls were in accordance with this guideline. Data on purity of Imexine FM were not reported.

In Vitro Mammalian Cell Gene Mutation Assay (*hprt* locus)

Guideline:	OECD 476
Cells:	L5178Y Mouse lymphoma cells
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	2-hydroxyethylamino-5-nitroanisole
Solvent:	DMSO
Batch:	0507391
Purity:	99%
Concentrations:	Experiment 1: 100 - 1000 µg/ml both without and with S9-mix Experiment 2: 100 - 1250 µg/ml without S9-mix 100 - 1000 µg/ml with S9-mix
Treatment	3 h treatment without and with S9-mix; expression period 7 days and selection period of 12 days
GLP:	in compliance

2-Hydroxyethylamino-5-nitroanisole was assayed for gene mutations at the *hprt* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Test concentrations were based on the results of a pre-test on toxicity measuring adjusted relative survival (relative survival corrected for loss of cells during the treatment period). In the main test, cells were treated for 3 h in the absence or presence of S9-mix followed by an expression period of 7 days to fix the DNA damage into a stable *hprt* mutation. Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was measured in the main experiments as adjusted relative survival of the

treated cultures relative to the total growth of the solvent control cultures. Negative and positive controls were in accordance with the OECD guideline.

Results
Measurements on post treatment media indicated that 2-hydroxyethylamino-5-nitroanisole had no effect on pH values or on osmolarity.
In experiment 1, precipitation of 2-hydroxyethylamino-5-nitroanisole occurred from 700 µg/ml. In experiment 2, from 800 µg/ml and above. The appropriate level of toxicity (10-20% survival after the highest dose) was reached in experiment 1 with S9-mix and in experiment 2 without S9-mix at the second highest dose tested. Excessive toxicity was seen at the highest dose. In experiment 1 without S9-mix and experiment 2 with S9-mix the appropriate level of toxicity was not reached eventually pointing to insufficient exposure of the cells.
No biological relevant, reproducible increase in the mutation frequency was observed following treatment with 2-hydroxyethylamino-5-nitroanisole at any dose level tested in the absence or presence of S9-mix, in both experiment 1 and 2.

Conclusion
Under the experimental conditions used, 2-hydroxyethylamino-5-nitroanisole was considered not mutagenic in the mouse lymphoma assay at the *hprt* locus.

Ref.: 7

Comment
In experiment 1 without S9 and experiment 2 with S9 the appropriate level of toxicity was not reached pointing to insufficient exposure of the cells. However, precipitation was observed at the time of exposure (but not at the end of the treatment incubation period) at the higher concentrations. As such the higher concentrations may be considered very close to the limit of precipitation, and this did not prejudice the validity of the study.

In Vitro **Micronucleus Test**

Guideline:	OECD 487 (draft 2004)	
Cells:	human lymphocytes from 2 healthy, non-smoking male volunteers	
Replicates:	duplicates in 2 independent experiments	
Test substance:	2-hydroxyethylamino-5-nitroanisole	
Solvent:	DMSO	
Batch:	0507391	
Purity:	> 99.5%	
Concentrations:	Experiment 1:	0, 225, 550 and 625 µg/ml (without S9-mix) 0, 600, 1050 and 1150 µg/ml (with S9-mix)
	Experiment 2:	0, 300, 700 and 860 µg/ml (without S9-mix) 0, 900, 1050 and 1250 µg/ml (with S9-mix)
Treatment	Experiment 1:	24 h PHA followed by 20 + 28 h treatment (without S9-mix) 24 h PHA followed by 3 + 45 h treatment (with S9-mix)
	Experiment 2:	48 h PHA followed by 20 + 28 h treatment (without S9-mix) 48 h PHA followed by 3 + 45 h treatment (with S9-mix)
GLP:	In compliance	

2-Hydroxyethylamino-5-nitroanisole has been investigated in the absence and presence of metabolic activation for the induction of micronuclei in cultured human lymphocytes. Suitable ranges of test concentrations for the main experiment were based on the results of a cytotoxicity range-finding experiment measuring replication index (RI). To determine the test concentrations for micronucleus analysis in each separate experiment, the RI is measured in cultures treated with increasing concentrations of 2-hydroxyethylamino-5-nitroanisole. The top dose for micronucleus analysis was to be the one at which at least

approximately 60% reduction in RI occurred or the highest dose tested. Two lower doses were selected so that a range of cytotoxicity from maximum (60%) to little or none is covered. Treatment periods were 20 h without and 3 h with S9-mix. Harvest times were 72 hours (experiment 1) or 96 hours (experiments 2) after the beginning of culture. The final 27-28 h of incubation was in the presence of cytochalasin B (at a final concentration of 6 µg/ml). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Negative and positive controls were in accordance with the draft guideline.

Results
Measurements on post-treatment media in the absence or presence of S9-mix indicated that 2-hydroxyethylamino-5-nitroanisole had no effect on osmolarity or pH as compared to concurrent vehicle controls.
Without metabolic activation, an increase in the number of micronucleated lymphocytes compared to concurrent control values was only found in the second experiment at the highest concentration tested. Since this increase fell within the range of historical vehicle controls it is considered not biologically relevant.
With metabolic activation, an increase in the number of micronucleated lymphocytes compared to concurrent control values was found in the first experiment at the highest dose and in the second experiment at the mid and high dose tested. The increase in experiment 1 fell within but those of experiment 2 outside the range of historical vehicle controls. The latter ones are, consequently, considered as biologically relevant.

Conclusion
Under the experimental conditions used 2-hydroxyethylamino-5-nitroanisole did induce micronuclei and, consequently, is genotoxic (clastogenic and/or aneugenic) in cultured human peripheral lymphocytes *in vitro*.

Ref.: 8

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Taken from SCCP/1099/07

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474
Species/strain:	Crl:CD (SD) rats
Group size:	5 rats/sex/group
Test substance:	2-hydroxyethylamino-5-nitroanisole
Batch:	0507391
Purity:	99.0%
Dose level:	500, 1000 and 2000 mg/kg bw
Route:	oral gavage, once
Vehicle:	0.5% aqueous methylcellulose
Sacrifice times:	24 h for all dose levels, vehicle control and positive control, 48 h for the highest dose and vehicle control.
GLP:	in compliance

2-Hydroxyethylamino-5-nitroanisole has been investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on a dose range finding assay in which clinical (toxic) signs and mortality were recorded. In the main experiment rats were exposed by gavage to single doses of 0, 500, 1000 and 2000 mg/kg bw 2-hydroxyethylamino-5-nitroanisole. Bone marrow cells were collected 24 h or 48 h (highest dose and vehicle control only) after dosing. Satellite rats (3 rats/sex) allocated for determination of plasma level of 2-hydroxyethylamino-5-nitroanisole (determined 0.5 and 2 h after treatment) were incorporated.

Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio). Moreover, all animals were observed immediately after dosing and at least daily for clinical signs and mortality. Bone marrow preparations were stained and examined microscopically for the PCE/NCE ratio and micronuclei. Negative and positive controls were in accordance with the OECD draft guideline.

Results
One male rat of the mid dose and one male rat of the high dose (24 h sacrifice group) were found dead one day after dosing. Clinical signs included hypoactivity, squinted eyes, flattened poisture laboured respiration, orange discoloration of various body parts, orange genital discharge and yellow urine. The latter two are considered to be evidence of systemic exposure to 2-hydroxyethylamino-5-nitroanisole. Exposure to 2-hydroxyethylamino-5-nitroanisole resulted in a decreased PCE/NCE ratio again indicating to exposure of the bone marrow cells.
2-Hydroxyethylamino-5-nitroanisole did not induce a biological relevant increase in micronucleated erythrocytes in any of the groups treated.

Conclusion
Under the experimental conditions used 2-hydroxyethylamino-5-nitroanisole did not induce micronuclei in bone marrow cells of treated rats and, consequently, 2-hydroxyethylamino-5-nitroanisole was not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of rats.
Ref.: 9

Comment
Although satellite rats (3 rats/sex) for determination of plasma level of 2-hydroxyethylamino-5-nitroanisole were incorporated in the experiment, plasma levels were not determined. This is acceptable since the clinical signs observed and the decrease in the PCE/NCE ratio indicate exposure of the bone marrow cells.

New study (submission III)

Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo*

Guideline:	OECD 486 (1997)
Species/strain:	rat, Wistar HsdCpb: WU (SPF)
Group size:	32 males
Test substance:	2-hydroxyethylamino-5-nitro-anisole
Batch:	0507391
Purity:	> 99.5% (HPLC°
Vehicle:	0.5% aqueous methylcellulose
Dose:	875 and 1750
Dose level:	10 ml/kg bw
Route:	oral (gavage), once
Positive control:	4h preparation interval: N,N'-dimethylhydrazinedihydrochloride (DMH) 16h preparation interval: 2-acetylaminofluorene (2-AAF)
GLP:	in compliance
Study period:	15 September – 23 October 2008

The test item was assessed in the *in vivo* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats.

The test item was formulated in 0.5% aqueous methylcellulose, which was used as vehicle control. The volume administered orally was 10 ml/kg bw. After a treatment period of 4 and 16 hours, respectively, the animals were anaesthetised and sacrificed by liver perfusion.

Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine), which is incorporated if UDS occurs.

The test item was tested at the following dose levels: 4 and 16 hours preparation intervals with 875 and 1750 mg/kg bw.

The highest dose (1750 mg/kg bw) was estimated in a pre-experiment to be the maximum applicable dose, at which clinical signs of toxicity occurred without affecting survival rates. In the main experiment one rat (animal no. 28) died after treatment with this dose.
The urine of the treated animals was discoloured, thus confirming bioavailability of the test item.
For each experimental group including the controls, hepatocytes from four treated animals were assessed for the occurrence of UDS.
The viability of the hepatocytes was not substantially affected by the *in vivo* treatment with the test item.
None of the tested dose levels revealed UDS induction in the hepatocytes of the treated animals as compared to the corresponding vehicle controls.
Appropriate reference mutagens [DMH, 80 mg/kg bw and 2-AAF, 100 mg/kg bw] were used as positive controls. Treatment with the positive control substances revealed distinct increases in the number of nuclear and net grain counts.

Conclusion
Under the experimental conditions reported, the test item did not induce DNA-damage leading to increased DNA synthesis in the hepatocytes of the treated rats. Therefore, 2-Hydroxyethylamino-5-nitro-anisole is considered to be non-genotoxic in this in vivo UDS test system.

Ref: 3 (subm III)

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Taken from SCCP/1099/07

Guideline:	OECD 414
Species/strain:	rats, Sprague-Dawley Ico:OFA.SD. (IOPS Caw)
Group size:	25 mated females per dose group
Test substance:	B052 suspended in 0.5% carboxymethylcellulose
Batch:	0507391
Purity:	99.0%
Dose:	0, 100, 250, 750 mg/kg bw/day
Route:	oral, gavage
Exposure:	day 6 to 19 of gestation
GLP:	in compliance

The test substance was administered to 25 mated females per dose group by oral gavage from 6 to 19 of gestation at the doses 100, 250 and 750 mg/kg bw/d, the controls received the vehicle 0.5% carboxymethylcellulose only. Mortality and clinical signs were evaluated

daily. Body weight and feed consumption were recorded on days 0, 6, 9, 12, 15, 18 and 20 of gestation. On day 20 the animals were sacrificed and necropsied and the foetuses were dissected. The common parameters were recorded and the foetuses where weighed, sexed and submitted to external examination. Approximately half of the foetuses were examined for visceral anomalies and skeletal abnormalities.

Results
8 females of the high dose group were found dead or were sacrificed moribund between day 8 and 20 of gestation. Except the low dose group all treated animals had yellow stained fur and yellow coloured urine. Isolated clinical signs were hairloss and subdued behaviour. Maternal body weight gain was severely reduced at 750 mg/kg bw/d accompanied by reduced feed consumption. The number of resorptions was not changed. Mean foetal weight was significantly reduced in the highest dose group. One external abnormality (acaudia) was found at 750 mg/kg bw/d which was considered an isolated finding. Visceral examination did not reveal further abnormalities. Parallel to the reduction in foetal weight in the high dose group incomplete ossification of os supraoccipitale, thoracic and sternal ossification centers occurred.

Conclusion
The NOAEL of maternal and embryo/foetal toxicity is 250 mg/kg bw/day.

Ref.: 10

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(2-Hydroxyethylamino-5-nitroanisole)

(Semi-permanent)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**1.05 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.735 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0,012 mg/kg**
No observed effect level (mg/kg) (90-day,oral,rat)	**NOAEL**	**=**	**100 mg/kg**
Margin of Safety	**NOAEL / SED**	**=**	**8333**

3.3.14. Discussion

Taken from SCCP/1099/07

This risk assessment relates to the use of 2-hydroxyethylamino-5-nitroanisole in non-oxidative hair dye formulations only.

Physico-chemical properties
2-Hydroxyethylamino-5-nitroanisole is used in semi-permanent hair dye formulations at a maximum concentration of 0.2%.
2-Hydroxyethylamino-5-nitroanisole is a secondary amine and thus is prone to nitrosation. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.
No documentation was submitted on the stability of the test substance in marketed products.

General toxicity
The maximal non-lethal dose of the test substance was 500 mg/kg bw and the minimal lethal dose is 1000 mg/kg bw under the experimental conditions of the acute oral toxicity study in rats.
The NOAEL of Imexine FM in a 90-day oral toxicity study in rats was 100 mg/kg bw/day.
The NOAEL for maternal and embryo/foetal toxicity was 250 mg/kg bw/d.

Irritation / sensitisation
Under the conditions of the study, a 10% dilution of 2-hydroxyethylamino-5-nitroanisole showed transient, slight irritant potential to rabbit skin.
1% 2-hydroxyethylamino-5-nitroanisole was non-irritating to rabbit eyes.
On the basis of the results of the murine LLNA, 2-hydroxyethylamino-5-nitroanisole at a maximal dilution of 10% was considered to have no skin sensitising potential.

Dermal absorption
As only a total of 7 chambers were used from a total of 3 donors, the dermal absorption of 2-hydroxyethylamino-5-nitroanisole incorporated at 0.21% in a typical semi-permanent hair

colouring formulation should be the maximum observed for use in MOS calculation: A_{max} 1.05 µg $_{eq}$ cm^{-2} (2.50% of the applied dose).

Mutagenicity
2-hydroxyethylamino-5-nitroanisole was sufficiently investigated in valid genotoxicity tests for the 3 types of genotoxic endpoints: gene mutations, structural and numerical chromosome mutations. 2-hydroxyethylamino-5-nitroanisole induced gene mutations in bacteria, but not in mammalian cells at the *hprt* locus of mouse lymphoma cells and did not induce DNA repair in the UDS assay *in vivo*. Although 2-hydroxyethylamino-5-nitroanisole induced chromosomal mutations in the *in vitro* micronucleus assay, this effect was not observed in a well performed *in vivo* micronucleus assay in rats. It can be concluded that 2-hydroxyethylamino-5-nitroanisole does not have genotoxic potential *in vivo*.

Carcinogenicity
No data submitted

4. CONCLUSION

This risk assessment relates to the use of 2-Hydroxyethylamino-5-nitroanisole in non-oxidative hair dye formulation only

The SCCS is of the opinion that the use of 2-Hydroxyethylamino-5-nitroanisole as an ingredient in non-oxidative hair dye formulations with an on-head concentration of 0.2% does not pose a risk to the health of the consumer.

2-Hydroxyethylamino-5-nitroanisole is a secondary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission III, April 2009

References in i*talics* were already submitted to the SCCS and not submitted as full reports in the present dossier.

1. *M.D. Kelly. Imexine FM: Bacterial Reverse Mutation Test; Four Salmonella typhimurium Strains; One Escherichia coli Strain. Toxicol Laboratories Study No. M/Ames/40257, 1994*
2. *M. Lloyd. 2-Hydroxyethylamino-5-Nitroanisole (B052): Mutation at the hprt locus of L5178Y Mouse Lymphoma Cells using the Microtitre® Fluctuation Technique. Covance Laboratories Study No. 413/89, 2005*
3. N. Honarvar. In vivo Unscheduled DNA Synthesis in Rat Hepatocytes with 2-Hydroxyethylamino-5-Nitroanisole (B052). Harlan Cytotest Cell Research Study No. 1210200, 2009

4. *G. Erexson. In vivo Rat Micronucleus Assay with 2-Hydroxyethylamino-5-Nitroanisole (B052). Covance Laboratories Study No. 6182-124, 2005*
5. *G. Clare. 2-Hydroxyethylamino-5-Nitroanisole (B052): Induction of micronuclei in cultured human peripheral blood lymphocytes. Covance Laboratories Study No. 413/90, 2005*

Taken from SCCP/1099/07

The references in italics (13-22) were not submitted by the applicant as full reports in the present dossier, since the studies reported therein were not considered to be adequate.

1. G. Sire. 2-Hydroxyethylamino-5-Nitroanisole (B052): Acute Oral Toxicity Study in Rats "Fixed Dose Method". CIT Safety & Health Research Laboratories Study No. 27930 TAR, 2004
2. G. Sire. 2-Hydroxyethylamino-5-Nitroanisole (B052) at the concentration of 10%: Acute Dermal Irritation in Rabbits. CIT Safety and Health Research Laboratories Study No. 27932 TAL, 2005
3. G. Sire. 2-Hydroxyethylamino-5-Nitroanisole (B052) at the concentration of 1%: Acute Eye Irritation in Rabbits. CIT Safety and Health Research Laboratories Study No. 27931 TAL, 2005
4. G. Sire. 2-Hydroxyethylamino-5-Nitroanisole (B052): Evaluation of Skin Sensitisation Potential in Mice Using the Local Lymph Node Assay (LLNA). CIT Safety and Health Research Laboratories Study No. 27933 TSS, 2005
5. S-A. Brownlie. Imexine FM: 13 Week Oral (Gavage) Toxicity Study in the Rat. Quintiles Study No. LRL/81/95, 1997
6. M.D. Kelly. Imexine FM: Bacterial Reverse Mutation Test; Four Salmonella typhimurium Strains; One Escherichia coli Strain. Toxicol Laboratories Study No. M/Ames/40257, 1994
7. M. Lloyd. 2-Hydroxyethylamino-5-Nitroanisole (B052): Mutation at the hprt locus of L5178Y Mouse Lymphoma Cells using the Microtitre® Fluctuation Technique. Covance Laboratories Study No. 413/89, 2005
8. G. Clare. 2-Hydroxyethylamino-5-Nitroanisole (B052): Induction of micronuclei in cultured human peripheral blood lymphocytes. Covance Laboratories Study No. 413/90, 2005
9. G. Erexson. In vivo Rat Micronucleus Assay with 2-Hydroxyethylamino-5-Nitroanisole (B052). Covance Laboratories Study No. 6182-124, 2005
10. P.C. Barrow. B052 - Prenatal developmental toxicity study by the oral route (gavage) in rats. MDS Pharma Services Study No. AA20578, 2005
11. R.A.F. de Ligt. In Vitro Percutaneous Absorption of [^{14}C]-2-hydroxyethylamino-5-nitro-anisole through human skin membranes using flow-through diffusion cells. TNO Study No. 6096, 2005
12. D. Mathieu. 2-Hydroxyethylamino-5-Nitroanisole (B052): Validation of the Analytical Method and Determination of Homogeneity and Stability of Dosage Forms. CIT Safety and Health Research Laboratories Study No. 27929 AHS, 2005
13. *D.R. Chubb. Imexine FM – Acute Oral toxicity Study in the Rat. Toxicol Laboratories Limited Study No. A/O36445, 1993*
14. *J.P. Guillot. Colorant N° 93: HydroxyEthyl Amino-2 Nitro-5 Anisole- IMEXINE FM N° de lot : DG 2- 30.76. Test for the Determination of Primary Cutaneous Irritation in the Rabbit. Institut Francais de Recherches et Essais Biologiques (IFREB) Report No. IFREB-R 802264, 1978*
15. *J.P. Guillot. Colorant N° 93: HydroxyEthyl Amino-2 Nitro-5 Anisole- IMEXINE FM N° de lot ; DG 2- 30.76: Test for the Determination of the Index of Ocular Irritation in the Rabbit. Institut Francais de Recherches et Essais Biologiques (IFREB) Report No. IFREB-R 801240 1978*
16. *J.P. Guillot. IFG 64/78- Colorant IMEXINE FM (Ref.: DG 2) : Evaluation of the Sensitizing Potential of a Test Substance by Topical Applications in the Guinea Pig.*

Institut Francais de Recherches et Essais Biologiques (IFREB) Report No. IFREB-R 808339, 1978

17. *C. Fabreguettes. Toxicity Study for 28 Days by Oral Administration (Gavage) to Rats-Imexine FM. Centre International de Toxicologie (CIT) Report No. 5718 TSR, 1990*
18. *M.M. Shahin. Mutagenic Evaluation of the Compound β-Hydroxyethylamino-2, Nitro-5-Anisole in Ames Salmonella Typhimurium/Microsome Plate test. Department of Chemical Protection and Photobiological Research in Vitro, Advanced Research Center – L'Oréal Study No. MMS 1 – 26/6/1978,, 1992*
19. *M.D. Kelly. In Vitro Cell Cytogenic Test in Chinese Hamster Lung (CHL) Cells. Imexine FM. Toxicol Laboratories Study No. M/CHL/36436, 1994*
20. *K.G. Dossou. Test du Micronoyau sur Moelle Osseuse de Souris traitée in Vivo par Voie Intrapéritonéale. Produit Testé: B 52 1-methoxy-2-β-Hydroxyethylamino-5-Nitro-Benzene. Département des Controles Biologiques – L'Oréal Report No. CS/DM/93/001, 1993*
21. *F. Bourcier. Assessment of Possible Embryotoxic or Teratogenic Effects by Oral route in Rats- IMEXINE FM. Centre International de Toxicologie (C.I.T.) Report No. 6465 RSR, 1990*
22. *M. Cottin. Pénétration du Colorant IMEXINE FM à travers l'Epiderme Humain Monte sur Cellules de Diffusion type Franz. Chimie Analytique-Controles Biologiques et Méthodes Alternatives- L'Oréal Reports 89/09/466 and 89/09/467, 1989*