EUROPEAN COMMISSION

Health & Consumer Protection
Directorate-General

Scientific Committee on Consumer Products

SCCP

OPINION ON
2-Chloro-6-ethylamino-4-nitrophenol

COLIPA n° B89

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

The SCCP adopted this opinion at its 14th plenary of 18 December 2007

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 Products (SCCP), 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).

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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Opinion to be cited as: SCCP (Scientific Committee on Consumer Products), Opinion on 2-chloro-6-ethylamino-4-nitrophenol, 18 December 2007

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1. BACKGROUND

Submission I for 2-chloro-6-ethylamino-4-nitrophenol was submitted in July 1992 by COLIPA[1,2].

The Scientific Committee on Cosmetology (SCC) adopted at its 62nd plenary meeting on 18 February 1996 an opinion (SPC/1394/93) with the final conclusion that:

"*The substance can be classified as non irritating to mucous membranes and as non sensitizer. In the 90-day studies with rats, 10 mg/kg bw is considered to be the NOAEL. In the teratogenicity study, no signs of maternal or foetal toxicity were observed after administration of 90 mg/kg bw in rats. It should be noted that the NOAEL stems from a daily exposure for 90 days, whereas human exposure to permanent hair dye is unlikely to be more frequent than once a month. Percutaneous absorption of a formulation was 0.14% without and 0.10% with H_2O_2. In general the test substance did not show any mutagenic potential.*"

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 55 on the List of substances, provisionally allowed, which cosmetic products must not contain except subject to restrictions and conditions laid down.

Submission II was submitted in July 2005 by COLIPA. According to this submission 2-chloro-6-ethylamino-4-nitrophenol is used as:

a) a non-reactive hair colouring agent ("direct dye") in non-oxidative hair dye formulations at a maximum on-head concentration of 3%. It is common practice to apply 35 to 50 g of the product over a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at weekly intervals.
b) a non-reactive hair colouring agent ("direct dye") in oxidative hair dye formulations at a maximum on-head concentration of 1.5%. The colorant component and a developer (hydrogen peroxide) are mixed in ratios between 1:1 to 1:3. It is common practice to apply up to 100 g of the finished mixed product for a period of 30 minutes followed by rinse off with water and shampoo. The application may be repeated at monthly intervals.

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider 2-chloro-6-ethylamino-4-nitrophenol safe for use as a non-oxidative hair dye with an on-head concentration of maximum 3.0% taken into account the scientific data provided?*

2. *Does the SCCP consider 2-chloro-6-ethylamino-4-nitrophenol safe for use in oxidative hair dye formulations with an on-head concentration of maximum 1.5% taken into account the scientific data provided?*

3. *Does the SCCP recommend any further restrictions with regard to the use of 2-chloro-6-ethylamino-4-nitrophenol in any non-oxidative or oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association
[2] According to records of COLIPA

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2-Chloro-6-ethylamino-4-nitrophenol (INCI)

3.1.1.2. Chemical names

Phenol, 2-chloro-6-(ethylamino)-4-nitro- (CA INDEX NAME, 9CI)
2-Chloro-6-(ethylamino)-4-nitrophenol (IUPAC)

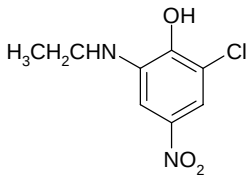
3.1.1.3. Trade names and abbreviations

Ethylchloroorange
Rot CO (COS 552)
COLIPA n° B089

3.1.1.4. CAS / EINECS number

CAS: 131657-78-8
ELINCS: 411-440-1 (Rot CO)

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_8H_9ClN_2O_3$

3.1.2. Physical form

Red-orange powder

3.1.3. Molecular weight

Molecular weight: 216.62

3.1.4. Purity, composition and substance codes

Composition (purity and impurities) of different batches of 2-Chloro-6-ethylamino-4-nitrophenol

Description of sample	AR917	GST 1-05079	9801090301 (R99005128)	4/01 (R0072415)	AR718	AR633	AR837
Date of Entry		07/11/91	08/12/99	01/23/02			
References of Analyses:	G 2004/002	G 2004/002	A 1999/400 G 2004/002	G 2004/002	G 2004/002	G 2004/002	G 2004/002
Characterisation /identification	NMR	NMR	NMR	NMR	NMR	NMR	NMR
NMR content / weight %	99.1	99.6	99.6	99.4	99.2	99.5	99.2
HPLC purity / area %**							
210 nm	99.8	99.9	99.8	99.7	99.9	99.8	99.9
254 nm	99.7	100	99.9	99.7	99.8	99.8	99.9
400 nm	99.8	100	99.9	99.6	99.8	99.9	99.8
HPLC content weight %	96.5	98.0		96.8	98.3	97.2	97.5
Content of 2-amino-6-chloro-4-nitrophenol / ppm	1241	163	490	659	933	969	876
Content of 6-chloro-2,4-dinitrophenol / ppm	< 13*	< 13*	< 13*	< 13*	< 13*	< 13*	< 13*
Water content / weight %	0.04	0.01	0.01	0.04	0.02	0.02	0.02
Loss on drying / weight %	0.06	0.03	0.03	0.07	°	0.05	0.04
Residue on ignition / weight %	0.05	0.11	0.01	0.02	0.11	0.06	0.09

* Below detection limit; indicated value shows detection limit.
** HPLC conditions: Purospher RP C18e 5µm 250 x 4 mm with precolumn; Eluent: 50 % acetonitrile / 50% 0,010M KH_2PO_4 buffer at pH3.5; Flow: 1 ml/min; 40°C
° Not determined because of lack of substance

Declaration by the applicant:
Batch 9801020182 from the current market production is not included in the batch analysis. However, batch 9801020182 was approved to fulfil the internal specification in the routine quality check and thus corresponds with the description of batch 9801090301.

3.1.5. Impurities / accompanying contaminants

See point 3.1.4 'Purity, composition and substance codes'

3.1.6. Solubility

Water:	104.7 mg/l (22°C)	(EU – A.6)	(*Reference: 8)*
Acetone/water (1:1):	2.6 weight % (pH 3.8)		
DMSO:	> 10 weight %		
Ethanol:	> 10 weight %		

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow} :	2.48 (pH 7.5 ; 25°C)	(EU – A.8)	(*Reference: 1*)

3.1.8. Additional physical and chemical specifications

Organoleptic properties: Red-orange powder
Particle size distribution: 201.8 µm (mean particle diameter; laser diffraction) (*Ref.: 2)*

pH-value:	4.0 – 6.0 (20°C ; 0.05 % aqueous solution)		
Melting point:	134.6 °C	(EU – A.1)	(*Ref.: 3)*
Boiling point:	138.8°C (decomposition)	(EU – A.2)	(*Ref.: 4)*
Relative density:	D_4^{20} = 0.549	(EU – A.3)	(*Ref.: 5)*
Vapour pressure:	5.7 hPa (20°C)	(EU – A.4)	(*Ref.: 6)*
Surface tension (in water):	72.3 mN/m (20.1°C)	(EU – A.5)	(*Ref.: 7)*
Flammability (solids):	not highly flammable	(EU – A.10)	(*Ref.: 9)*
Explosive properties:	not explosive	(EU – A.14)	(*Ref.: 10)*
Relative self-ignition temperature:	400°C	(EU – A.16)	(*Ref.: 11)*
Oxidising properties:	not oxidising	(EU – A.17)	(*Ref.: 12*)

UV-VIS Spectra (200-800nm): absorption maxima at 207 nm, 280 nm and 453 nm

3.1.9. Homogeneity and Stability

2-Chloro-6-ethylamino-4-nitrophenol is described to be stable for >5 years when stored in dryness and dark.

Stability of 2-Chloro-6-ethylamino-4-nitrophenol solutions stored up to 7 days at room temperature and in the absence of light:

Approximately 10% (w/w) solution in DMSO: changes in concentration 97.4 -93.9%
Approximately 2% (w/w) solution in acetone water (1:1): changes in concentration 94.8 -99.4%
Approximately 0.09% solution in water (pH 5.8): changes in concentration 97.5 -97.9%

Freshly prepared test solutions of 2-Chloro-6-ethylamino-4-nitrophenol prepared in 0.05% CMC were used within 6 hours. The concentration of the test substance determined by photometry varied 2.5-8.9% of the theoretical concentration.

General Comments to physico-chemical characterisation

- No documentation is provided for the characterisation and composition of various batches of 2-Chloro-6-ethylamino-4-nitrophenol
- Stability of 2-Chloro-6-ethylamino-4-nitrophenol in marketed products is not provided.
- 2-Chloro-6-ethylamino-4-nitrophenol is a secondary amine, and thus it is prone to nitrosation. Nitrosamine content in 2-Chloro-6-ethylamino-4-nitrophenol is not reported.

3.2. Function and uses

2-Chloro-6-ethylamino-4-nitrophenol is used as an ingredient in oxidative hair dye formulations at a maximum on-head concentration of 1.5%.
The colorant component and a developer (hydrogen peroxide) are mixed at ratios between 1+1 to 1+3.

2-Chloro-6-ethylamino-4-nitrophenol is used as a hair colouring agent in semi-permanent hair dye formulations at a maximum on head concentration of 3%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401
Species/strain:	Rat, strain Wistar Crl.:Wi/Br (SPF)
Group size:	5 per sex and dose
Test substance:	*2*-Chloro-6-ethylamino-4-nitrophenol (CEN) in 0.5% Carboxymethylcellulose
Batch:	AR 837
Purity:	99.9%
Dose levels:	1000, 1500, 2000 and 2500 mg/kg bw
Route:	Oral, gavage
Exposure:	Single administration
GLP:	In compliance

2-Chloro-6-ethylamino-4-nitrophenol (CEN) was suspended in 0.5% carboxymethylcellulose and administered once by gavage to 5 female and 5 male Wistar rats each at doses of 1000, 1500, 2000 and 2500 mg/kg bw. All animals that died within the observation period of 14 days were examined macroscopically. All animals that survived until the end of the observation period were necropsied and examined macroscopically.

Results
Mortalities occurred within the first 6 h in all dose groups with the following frequency: 9/10 animals in the 2500 mg/kg bw group, 6/10 animals at 2000 mg/kg bw, 3/10 at 1500 mg/kg bw and 1/10 at 1000 mg/kg. Necropsy findings included an orange-red discolouration of spleen, liver, kidney and serosa. Body weight gain in the surviving rats was not affected and the terminal necropsy revealed no unusual findings.
Treatment-related effects noted were reduced activity with apathy, ataxia with accordant anomalies in posture, muscle hypotonia, piloerection. The intensity of the observed effects increased with the dosage and the effects became visible about 20 h post-application and lasted until 48 h post application. Recovery of survivors was complete.

Conclusion
The acute median lethal oral dose (LD_{50}) of *2*-chloro-6-ethylamino-4-nitrophenol was calculated as: 1461 mg/kg bw (females), 2026 mg/kg bw (males) and 1728 mg/kg bw (both sexes).

Ref.: 14

3.3.1.2. Acute dermal toxicity

Guideline:	OECD 402
Species/strain:	Rat, strain Sprague Dawley, Him:OFA
Group size:	5 per sex
Test substance:	*2*-chloro-6-ethylamino-4-nitrophenol dissolved in distilled water at a ratio of 1:1 (w/w)
Batch:	AR 917
Purity:	99.7%
Dose level:	2000 mg/kg bw
Route:	Dermal
Exposure:	Single administration
GLP:	In compliance

2-Chloro-6-ethylamino-4-nitrophenol suspended in distilled water was applied to a clipped area (5 cm x 6 cm) on the dorsal thoracic region of 5 male and 5 female rats. The area was then covered by a cellulose patch for 24 h. Residual test substance was wiped off with wet cellulose tissues after 24 h application. Clinical observation was performed 10 min, 30 min and 1 h and at one hour intervals until 6 h per application. During the observation period of 14 days clinical observations were recorded daily. Body weights were determined before substance application and on days 7 and 14.

Results
All animals survived until the end of the study. Body weight gain was not affected by the treatment. The only effects noted were a transient diarrhoea in 3/5 males and 4/5 females one day after the treatment and chromodacryorrhoea which may be attributed to stress. Staining of the fur and tail was observed in all animals. At necropsy no treatment related or toxicologically relevant findings were noted.

Conclusion
The acute median lethal dermal dose (LD_{50}) of *2*-chloro-6-ethylamino-4-nitrophenol was > 2000 mg/kg bw in rats for both sexes.

Ref.: 15

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Skin irritation in rabbits (undiluted substance)

Guideline:	OECD 404
Species/strain:	Rabbit, strain New Zealand White
Group size:	6, sex not indicated
Test substance:	ROT CO
Batch:	/
Purity:	/
Dose level:	0.5 g
Route:	Dermal
Application conditions:	Single administration, 4h, occlusive
GLP:	In compliance

0.5g of ROT CO was applied to two shaved areas (intact and scarified) of about 2.5 cm x 2.5 cm each on the back of 6 New Zealand White rabbits. After 4 h under occlusive patch conditions, the test item was washed off with lukewarm water. Skin reactions were evaluated after 30 min, 60 min, 24 h, 48 h and 72 h.

Results
No reactions (scores) of any type were observed at any time point on either intact or scarified skin.

Conclusion
ROT CO applied undiluted in solid form is not irritating to intact or abraded skin of rabbits after 4 hours occlusion.

Ref.: 16

Skin irritation in rabbits (diluted substance)

Guideline:	OECD 404
Species/strain:	Rabbit, strain New Zealand White
Group size:	6, sex not indicated
Test substance:	COS 552 ROT CO
Batch:	/
Purity:	/
Dose level:	0.5 ml of a 3% solution in propylene glycol (pH 3.7)
Route:	Dermal
Application conditions:	Single administration, 4h, occlusive
GLP:	In compliance

0.5 ml of a 3% solution of COS 552 ROT CO in propylene glycol were applied to two shaved areas (intact and scarified) of about 2.5 cm x 2.5 cm on the back of New Zealand White rabbits. After 4 h under occlusive conditions, the test item was washed off with lukewarm water. Skin reactions were evaluated after 30 min, 60 min, 24 h, 48 h and 72 h.

Results
No reactions (scores) of any type were observed at any time point on either intact or scarified skin.

Conclusion
COS 552 ROT CO applied as 3% solution in propylene glycol is not irritating to intact or abraded skin of rabbits after 4 hours occlusion.

Ref.: 17

3.3.2.2. Mucous membrane irritation

Eye irritation in rabbits (undiluted substance)

Guideline:	According to OECD 405
Species/strain:	Rabbit, strain New Zealand White
Group size:	2 groups of 3 animals, sex not indicated
Test substance:	ROT CO
Batch:	/
Purity:	/
Dose level:	0.1 g
Route:	Ocular
Application conditions:	Single administration, rinsing/non-rinsing in 3 animals each
GLP:	In compliance

0.1 g of ROT CO was instilled into the conjunctival sack of the left eye of 6 New Zealand White rabbits. The right eyes served as controls. In three animals the treated eyes were rinsed with lukewarm water 4 seconds after application. Evaluation was performed 1, 24, 48 and 72 h after the application. After 24 h and 72 h an additional examination using 1% fluorescein solution was performed. Evaluation of corneal, iridial and conjunctival lesions was undertaken.

Results
No corneal lesions were noted at any reading.
One hour after substance application slight (score 1 in 4/6 animals) to moderate (score 2-3 in 2/6 animals) conjunctival redness and chemosis were noted. In two animals (in one with and in one without rinsing) a slight iridial effect (score 1) was also observed.
At 24 hours slight iridial effects were noted in 2/3 animals without and 1/3 animal with rinsing. Moderate conjunctival effects were noted in all 3 animals without and in one animal with rinsing. Iridial effects vanished completely within 48 hours. After 72 hours no

conjunctival reactions were noted besides a score 1 redness in one animal without rinsing. Considering the gradient of the results, it was assumed that these effects were reversible.

Conclusion
Undiluted ROT CO was irritating to rabbits eyes.

Ref.: 18

Eye irritation in rabbits (diluted substance)

Guideline:	OECD 405
Species/strain:	Rabbit, strain New Zealand White
Group size:	3, sex not indicated
Test substance:	COS 552 ROT CO
Batch:	/
Purity:	/
Dose level:	0.1 ml of a 3% solution in propylene glycol (pH 3.7)
Route:	Ocular
Application conditions:	Single administration, rinsing/non-rinsing in 3 animals each
GLP:	In compliance

0.1 ml of a 3% solution of COS 552 ROT CO in propylene glycol was instilled into the conjunctival sack of the left eye of 6 New Zealand White rabbits. The right eyes served as controls. In three animals the treated eyes were rinsed with lukewarm water 4 seconds after application. Evaluation was performed 1, 24, 48 and 72 h after the application. After 24 h and 72 h an additional examination using 1% fluorescein solution was performed. Evaluation of corneal, iridial and conjunctival lesions was undertaken.

Results
In the 3 animals without rinsing a slight conjunctival redness was observed 1 h after application. No other effects were observed at any reading with or without rinsing.

Conclusion
COS 552 ROT CO dissolved in propylene glycol at 3% showed transient irritation in a rabbit eye.

Ref.: 19

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species:	Mouse, strain CBA/J
Group size:	5 females per dose
Test substance:	2-chloro-6-ethylamino-4-nitrophenol (WR 23192)
Batch:	9801090301
Purity:	99.9%
Concentrations:	0.5, 1.5, 5 and 10% in DMSO (dimethylsulfoxide) 0.5, 1.5, 2 and 2.5% in water/acetone (1:1) mixed with olive oil at a ratio of 4:1
Route:	Dermal
Dosing schedule:	Once daily on three consecutive days
GLP:	In compliance

The skin sensitising potential of 2-chloro-6-ethylamino-4-nitrophenol was investigated in CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear.

25 µl of 0 (vehicle only), 0.5, 1.5, 5 and 10% of 2-chloro-6-ethylamino-4-nitrophenol in DMSO and 0, 0.5, 1.5, 2 and 2.5% in a mixture of water/acetone (1:1) with olive oil (4:1) (equal to the maximum solubility) were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. As positive control, p-phenylenediamine (PPD) at 1% in DMSO was investigated in parallel under identical test conditions.

On day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 23.6 µCi of [H^3] methyl thymidine. Approximately five hours later, the mice were killed by CO_2-inhalation and the draining auricular lymph nodes were removed and weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [H^3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).
The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
The stimulation indices in the groups with DMSO used as vehicle were:

1.1 (0.5% test group)
2.3 (1.5% test group)
4.2 (5% test group)
3.9 (10% test group)

Based on the findings with the two highest test concentrations (stimulation index exceeds the control figure by a factor ≥ 3) 2-chloro-6-ethylamino-4-nitrophenol is evaluated as skin sensitiser if applied in DMSO. The EC3 value calculated for the DMSO – vehicle group was 2.79%.

The stimulation indices in the groups with acetone/aqua/olive oil used as vehicle were:

1.2 (0.5% test group)
1.5 (1.5% test group)
1.5 (2.0% test group)
1.9 (2.5% test group)

With this vehicle no indication for skin sensitising potential was noted. A calculation of an EC3 value is not possible in case of the acetone/aqua/olive oil-vehicle groups.

The sensitivity of the test system was demonstrated by the reaction to p-phenylenediamine (1%) with a stimulation index of 7.0.

Conclusion
According to the results of this LLNA, 2-chloro-6-ethylamino-4-nitrophenol revealed moderate skin sensitising properties when applied in DMSO.

Ref.: 20

Maximisation (Magnusson and Kligman) Test

Two further skin sensitisation tests, Maximisation tests according to Magnusson and Kligman, have been performed with 2-chloro-6-ethylamino-4-nitrophenol.

In one assay, using a low test concentrations (1% in water for the dermal induction and challenge), no skin sensitising potential was noted.

In the other assay, a higher test concentration (25% in white petrolatum) was applied and caused discolouration of the application sites. As a standard scoring was compromised by this discolouration, evaluation was based on histopathological investigations and suggested a skin sensitising potential.

As both of tests have limitations either with regard to the test performance or due to the fact that unspecified test material was used, these tests are not discussed.

3.3.4. Dermal / percutaneous absorption

Percutaneous absorption *in vitro*

Guideline:	OECD Draft guideline "428" (2000)
Tissue:	female Porcine back skin (thickness: 898 ± 128 µm); 1 donor
Method:	Diffusion Teflon-chambers
Test substance:	ROT CO (WR23192) tested at a concentration of 3 % in a non-oxidative hair dye formulation
Batch:	9801020182
Purity:	99.9%
Application conditions:	3 mg/cm² tested as part of formulation
No. of chambers:	6 (five for the formulation containing the test item and one for the blank formulation)
GLP:	In compliance

The skin absorption of ROT CO (WR23192) at the maximum concentration intended for hair colorants (3%), was investigated with pig skin (Schweizer Edelschwein, 1 female donor) prepared from the back and the flanks (898 ± 128 µm). 3 mg of the dye was applied once to the skin in a commercial non-oxidative hair dye formulation (400 mg aqueous cream formulation containing 3% dye applied to 4 cm² skin).

The integrity of the skin was monitored at the beginning of the experiment using tritiated water.

A diffusion teflon chamber was used. The receptor solution (physiological phosphate buffer containing NaCl and antibiotics) was pumped through the receptor chamber at a rate of 5 ml/h. Six chambers were investigated.

Thirty minutes after substance application, the test item was removed by washing the skin twice with 4 ml water, then once with 4 ml washing solution (shampoo-formulation diluted with water) and again twice with water. The washing solutions were combined and the amount of dye was determined by HPLC.

Fractions of the receptor fluid were collected after 16, 24, 40, 48, 64 and 72 hours, concentrated directly after the pump and analysed immediately. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the dye content was quantified by means of HPLC.

Results
All samples/tissue extracts were analysed by HPLC. Based on the data for solubility (> 10% in DMSO, 0.74 mg/ml in receptor fluid) and stability (≥ 7 days in water-based systems and DMSO) provided in the report as well as based on results obtained in the pre-experiments for determination of the recovery from the different fractions/tissues, the substance is regarded to be stable for the given test procedure and at the detected levels. The limit of quantification of the applied method was 3 ng/HPLC-injection. For fractions of receptor fluid

in which quantification by HPLC was not possible (three were below LOQ) a theoretical maximum content of 6.2 ng/cm² was calculated, based on LOQ, recovery rate, etc.
The integrity of each skin sample was demonstrated with tritiated water, resulting in penetration rates of 1.02 to 1.36% of the applied dose. These figures were within the limit of acceptance (≤ 1.5%).

The total recovery of 99.1 ± 1.6% of the applied dose confirmed the validity of the test.
The majority of the applied dose of ROT CO (WR23192) remained on the skin surface representing 99.0 ± 1.6%.

At 72 hours 1.64 ± 0.296 µg/cm² (0.0548 ± 0.0093% of the applied dose) was recovered from the upper dermis and receptor fluid. The maximum amount considered available was 2.1µg/cm² (0.069% of the applied dose).

Conclusion
Under the described test conditions (single application for 72 hours duration), a maximum skin penetration rate (A_{max}) of 2.1µg/cm² (0.069% of the applied dose) of ROT CO (WR23192*)* was obtained by summing up the amounts for receptor fluid and for the lower skin compartment (upper dermis).

Ref.: 21

Comment
The number of replicates used in this study (1 donor, 5 chambers) was not according to the SCCP Notes of Guidance. The test was only performed under non-oxidative conditions. The study cannot be accepted.

Percutaneous absorption *in vivo* (see point 3.3.9. Toxicokinetics)

In the *in vivo* toxicokinetics study in rats percutaneous absorption of 2-chloro-6-ethylamino-4-nitrophenol was determined as 0.1% (2.16 µg/cm²) and as 0.14% (2.51 µg/cm²) of the amount of radioactivity applied dermally as part of oxidative (in the presence of hydrogen peroxide) and non-oxidative hair dye formulations, respectively. In this study, a concentration of only 2% was used whereas the intended concentration is 3%. Therefore, for the calculation of the Margin of Safety, a value of 2.51 µg/cm² was extrapolated (times 1.5 = 3.77 µg/cm²)

Ref.: 33

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

Guideline:	OECD 408
Species/strain:	Rat, strain Wistar, Crl:Wi/Br (SPF)
Group size:	15 per sex and dose 10 per sex (satellite recovery control and high dose groups)
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Batch:	AR 917
Purity:	99.7%
Dose levels:	10, 30 and 90 mg/kg bw in 0.5% carboxymethylcellulose (CMC)
Route:	Oral, gavage
Exposure:	Once daily over a period of 13 weeks

GLP: In compliance

Doses of 10, 30 and 90 mg/kg bw 2-chloro-6-ethylamino-4-nitrophenol in distilled water with 0.5% CMC were administered daily to groups of 15 male and 15 female rats by gavage in a total volume of 10 ml/kg bw over 13 weeks. A control group received the same dose volume of the vehicle (10 ml/kg, 0.5% CMC) daily throughout the exposure. Satellite groups consisting of an additional 10 males and 10 females for the control and the high dose groups were investigated after a 4-week treatment-free period.
Mortality, clinical signs, body weight and food consumption were recorded. An ophthalmologic investigation and an evaluation of hearing and reflexes with special regard to awareness, emotion, co-ordination and autonomic functions was performed before treatment, after 6 weeks, at the end of the treatment period and at the end of week 17 (recovery groups) in 10 animals per sex and dose.
Haematology, blood/clinical biochemistry in 10 and urinalysis in 5 animals per sex and dose group were performed after 6 and 13 weeks in all dose groups as well as after 17 weeks in the recovery groups.
All animals were subjected to a detailed necropsy and a number of organs (adrenals, brain, heart, kidneys, lung, liver, ovaries, pituitary and prostate gland, spleen, testes with epididymis, thymus, uterus) were weighed and the tissues and organs were fixed and stored for possible further examinations.
Organs and tissues of the control and high dose animals were examined histopathologically and all gross lesions noted were examined microscopically.

Results
No mortalities occurred and habit, motor and sensor activity were not influenced by the treatment. Reflex examinations, ophthalmologic investigations and hearing tests did not reveal treatment-related changes. No treatment-related gross abnormalities were observed during necropsy. The urine was discoloured throughout the study but food consumption was not affected.
In the high dose group body weight gain in male was significantly reduced. Haematological examinations revealed no treatment-related effects. The slight increase of reticulocyte and leucocyte counts in high dosed females at termination was still within the normal range.
Biochemical parameters were similar in control and treatment groups except for a slight and dose-related increase in total bilirubin and uric acid values which were, however, within the normal range.
No gross pathology lesions were noted at necropsy. Liver weights both corrected and uncorrected were increased significantly in a dose-related manner in the male animals in 30 and 90 mg/kg bw groups. Kidney weights in males were slightly increased but did not reach statistical significance. All the findings were reversible during the 4-week recovery period.
The histomorphological examination of the organs of high dose group did not reveal morphological changes related to the treatment. The alterations observed in different organs were considered to be similar in treated and control animals with regard to type, incidence and severity.

Conclusion
Due to significantly increased liver weight of male rats seen at 30 and 90 mg/kg bw after the 90 days of exposure, a NOEL of 10 mg/kg bw was deduced.

Ref.: 23

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Study 1

Guideline:	OECD 471 (1986)
Species/strain:	*Salmonella typhimurium,* strains TA98, TA100, TA 1535, TA1537, TA1538
Replicates:	3 replicates in three independent experiments
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Solvent:	DMSO
Batch:	1-05079
Purity:	100% (area)
Concentrations:	0, 0.96, 4.8, 24, 120 and 600 µg/plate (experiment 1) 0, 62.5, 125, 250, 500 and 1000 µg/plate (experiments 2 and 3)
Treatment:	Standard plate incorporation assay with and without S9-mix
GLP:	In compliance

2-chloro-6-ethylamino-4-nitrophenol dissolved in DMSO was tested for mutagenicity in the reverse mutation assay on bacteria in three independent plate incorporation assays. The *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 were exposed to the test substance at concentrations ranging from 0.96 µg/plate to 1000 µg/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. Toxicity was measured as a reduction in revertants and background lawn. Appropriate positive and negative (DMSO) controls were included according to OECD guidelines.

Results
In the main experiments toxic effects were measured as a reduction in the number of revertants. In experiment 1 toxic effect was noted in TA1537 at the highest tested concentration (600 µg/plate) both in the presence and absence of S9-mix. In experiment 2 and three toxic effects were noted in all tester strains in the presence and absence of S9-mix at 1000 µg/plate and at 500 µg/plate in TA1535 and TA1537 (exp 2 and 3) and TA 1538 (exp 3).

In experiment one, a concentration-related increase was observed in TA98 with and without metabolic activation up to more than a doubling of the negative control rate. In TA 100 both with and without metabolic activation a concentrated related increase was observed but a doubling of the spontaneous mutation rate was not achieved at any of the tested concentrations.

In experiment two, a significant and concentration-related increase was observed up to toxic concentrations in TA100 without and in TA1538 with metabolic activation and in TA98 both with and without metabolic activation. In experiment three a significant and concentrated related increase was observed in TA100 without metabolic activation, in TA98 with and without metabolic activation and finally in TA1538 without metabolic activation.

Conclusion
Under the test conditions reported, 2-chloro-6-ethylamino-4-nitrophenol did induce gene mutations in bacteria.

Ref.: 24

Comment

The historical data for positive controls and for negative controls without S9 were not included.

Study 2

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium*/strains TA98, TA100, TA 1535, TA1537, TA102
Replicates:	3 replicates in two independent experiments
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Solvent:	DMSO
Batch:	9801090301 (R99005128)
Purity:	99.9 area % (HPLC)
Concentrations:	0, 3, 10, 33, 100, 333, 1000, 2500 and 5000 μg/plate in both experiments
Treatment:	Experiment 1: Direct plate incorporation assay Experiment 2: Preincubation assay
GLP:	in compliance

2-chloro-6-ethylamino-4-nitrophenol dissolved in DMSO was tested for mutagenicity in the reverse mutation assay on bacteria (experiment 1: plate incorporation method, experiment 2: pre-incubation method) both with and without metabolic activation (S9-mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar Hanlbm rats). The *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to the test substance at concentrations ranging from 3 μg/plate to 5000 μg/plate with and without S9-mix. Test concentrations were selected based on the results obtained in a pre-experiment with strains TA98 and TA100. Positive and negative controls were included according to OECD guidelines.

Results
Reduced background growth was observed at higher test concentrations (≥ 1000 μg/plate) in the presence and absence of S9-mix in all strains investigated.
Distinct toxic effects, evident as a reduction in the number of revertants was noted in all tester strains without S9-mix at concentrations ≥ 333 μg/plate (TA102, experiment I), ≥ 1000 μg/plate (TA100 in experiment I; TA1537 and TA102 in experiment II), ≥ 2500 μg/plate (TA98 and TA1535 in experiment I; TA98, TA100 and TA1535 in experiment II), as well as 5000 μg/plate (TA1537, experiment I). With S9-mix a reduced number of revertants was noted in all tester strains at concentrations ≥ 1000 μg/plate (TA100, TA102 and TA1537, experiment I), ≥ 2500 μg/plate (TA100 and TA102 in experiment II; TA98 and TA1535 in experiment I + II), as well as at 5000 μg/plate (TA1537, experiment II).
There were no signs of a reproducible increase in revertant colony numbers in any of the five tester strains at any concentration either in the presence or in the absence of metabolic activation. Slightly higher mutation rates compared to the historical control range were noted in TA 102 for the solvent control (experiment I, with S9-mix) and the negative control (both experiments with and without S9-mix). The positive control in strain TA102 (experiment I) slightly exceeded the historical control range in the presence and absence of S9-mix. These deviations are considered within normal biological fluctuations in the number of colonies and are unlikely to have an important impact on the outcome of the study.

Conclusion
Under the test conditions reported, 2-chloro-6-ethylamino-4-nitrophenol did not induce gene mutations in any of the tester strains in the presence or absence of metabolic activation.

Ref.: 25

Comment

Several further Ames tests have been performed with 2-chloro-6-ethylamino-4-nitrophenol, most of them were not in compliance with the actual OECD guideline, were not performed according to GLP or unspecified material was used. Some assays revealed a mutagenic response mainly in TA98 and/or TA1538 and T100 in the absence of S9-mix, whereas no mutagenic effects were noted in others.

In vitro Mammalian Cell Gene Mutation Test (*tk*$^{+/-}$ locus)

Guideline:	OECD 476 (1997)	
Species/strain:	Mouse lymphoma cell line L5178Y (*tk* locus)	
Replicates:	duplicates in two independent experiments	
Test substance:	2-chloro-6-ethylamino-4-nitrophenol	
Solvent:	DMSO	
Batch:	9801090301 (R99005128)	
Purity:	99.9% (HPLC)	
Concentrations:	Experiment 1:	without metabolic activation (3 h treatment): 300, 400, 500, 600, 700, 800 μg/ml with metabolic activation (3 h treatment): 25, 30, 37.5, 45, 52.5, 60 μg/ml
	Experiment 2:	without metabolic activation (24 h treatment): 2.5, 5, 10, 20, 30, 40, 50 μg/ml with metabolic activation (3 h treatment): 50, 60, 70, 80, 90, 100, 120 μg/ml
Treatment:	Experiment 1:	3 h treatment with and without S9-mix, 48h expression period, selection growth 8 days
	Experiment 2:	3 h treatment with S9-mix, 48h expression period, selection growth 7–15 days
	24 h treatment without S9-mix, 48h expression period, selection growth 8 days	
GLP:	In compliance	

The test substance was examined for its genotoxic potential in the L5178Y TK$^{+/-}$ mouse lymphoma test in the absence and presence of metabolic activation (Aroclor induced rat liver S9-mix). A range-finding test (pre-test on toxicity, measuring relative suspension growth) and two independent mutagenicity experiments were carried out. The concentrations for Experiment 1 and 2 were selected based on the results of the range-finding experiment. Culture medium and DMSO were used as negative and solvent controls, while 4-nitroquinoline-1-oxide (NQO; 0.05 – 0.2 μg/ml) and benzo(a)pyrene (BP; 2.0 and 3.0 μg/ml) were used as positive controls in experiments without and with metabolic activation system, respectively. Two parallel cultures were investigated for each concentration and test group. Mutant frequency and cell survival (measured as cloning efficiency) were determined in parallel, and the ratio of small versus large colonies was calculated.

Results
Precipitation of test item was noted at the highest concentration (2166.3 μg/ml) in the absence and the presence of metabolic activation. In the range-finding study extreme toxicity (<10 % relative total growth, RTG) was observed with metabolic activation at ≥ 1000 μg/ml, thus the doses selected for the main study was 50 to 800 μg/ml. Without metabolic activation extreme toxicity was observed at all doses tested (62.5 – 2166.3 μg/ml), thus the doses selected for the main study was 2.5 to 60 μg/ml. In the cytoxicity range-finding study (24 h treatment), doses were tested without metabolic activation ranging from 7.8 to 2166.3 μg/ml. Extreme toxicity (<10 %) was observed at ≥ 125 μg/ml. The highest dose tested was at 62.5 μg/ml that yielded 11% RTG.
In the main experiments no precipitation of the test item was observed at the dose levels tested.

In experiment 1 no toxic effects were observed in both cultures at the highest concentration of 800 µg/ml without and 60 µg/ml with metabolic activation. In experiment 2 toxic effects occurred at 40 and 50 µg/ml in the absence of metabolic activation. Extreme toxicity (<10 %) was not observed at any doses with metabolic activation.
In experiment 1, a statistically significant increase in mutant frequency ($p<0.05$) was observed at the maximum tested dose (60 µg/ml) in the presence of metabolic activation. The increase provided a statistically significant linear trend ($p<0.01$). Treatments were performed up to a maximum treatment dose that yielded 37% RTG, and was therefore below the optimal toxicity limiting dose level of 10-20% RTG. Without metabolic activation a weak statistically significant linear trend was observed, but there were no significant increases in the mutation frequency at any doses tested.
In experiment 2 with metabolic activation, there were clear dose-related increases in mutant frequency that were statistically significant and a strong significant linear trend was observed. Without metabolic activation there was no linear trend and no significant increases in the mutation frequency at any doses tested.
At the concentrations of which a statistically significant increase in the mutant frequency was noted, increases in both small and large colony mutant frequencies were observed compared to the concurrent negative controls.

Conclusion
There were no signs of induced mutations without metabolic activation, using either 3 h or 24 h treatment periods. With metabolic activation mutations were induced. In experiment one, only the highest concentration induced a significant increase in the mutant frequency. However, higher concentrations were not tested although the level of toxicity required by the guideline was not reached. In experiment two, a very clear concentration dependent induction of mutations was measured. It is concluded that 2-chloro-6-ethylamino-4-nitrophenol is genotoxic (mutagenic and clastogenic) in the presence of metabolic activation, when tested under the conditions used in this study.

Ref.: 26

Comment
In a previous submission, a mouse lymphoma assay was described that was performed with 2-chloro-6-ethylamino-4-nitrophenol in 1987. As this test has several limitations (e.g. not in line with the actual guideline, insufficient level of toxicity, no colony sizing, inadequate reporting, unspecified material used etc.) the study is not presented here in detail. Result: negative.

In vitro Micronucleus Test

Guideline:	draft OECD 487	
Species/strain:	Human peripheral blood lymphocytes from two male donors	
Replicates:	Two cultures per concentration and positive controls (4 for the negative control). Two independent experiments	
Test substance:	2-chloro-6-ethylamino-4-nitrophenol	
Solvent:	DMSO	
Batch:	9801090301 (R99005128)	
Purity:	99.9% (HPLC)	
Concentrations:	Experiment 1:	with metabolic activation: 96.76, 189 and 461.4 µg/ml without metabolic activation: 28.24, 43.05, 53.15 µg/ml
	Experiment 2:	with metabolic activation (3h treatment+45h recovery): 430.5, 656.1, 810 µg/ml without metabolic activation (20h treatment+28h recovery): 43.05, 47.83, 59.05 µg/ml

Treatment:	Experiment 1:	24h mitogen stimulation followed by 3h treatment + 45h recovery period (+ S9-mix) 24h mitogen stimulation followed by 20h treatment + 28h recovery period (- S9-mix)
	Experiment 2:	48h mitogen stimulation followed by 3h treatment + 45h recovery period (+ S9-mix) 48h mitogen stimulation followed by 20h treatment + 28h recovery period (- S9-mix)
GLP:	In compliance	

2-chloro-6-ethylamino-4-nitrophenol dissolved in DMSO was examined for its clastogenic and aneugenic activity in the in vitro micronucleus assay using duplicate human lymphocyte cultures. Two independent assays were performed with and without metabolic activation (S9-mix from the liver of Aroclor induced rats).

In experiment 1, cells were treated with the test item 24 hours after mitogen stimulation with phytohaemagglutinin (PHA). In the second experiment, the treatment started 48 hours after the mitogen stimulation. The exposure times for the test item in the presence and the absence of S9-mix were 3 and 20 hours, respectively. The recovery periods were 28 and 45 hours for the test without and with S9-mix. For this period, cytochalasin B was added to the cultures at 6 µg/ml to block the cell but not the nuclear division. To calculate the replication index (RI), 500 cells per replicate (1000 per dose) were examined for proportions of mononucleate, binucleate and multinucleate cells. 1000 binucleate cells from each culture (2000 per dose) were analysed for the occurrence of micronuclei. The number of cells containing micronuclei and the number of micronuclei per cell was noted for each slide.
A broad concentration range (from 5.278 to 2200 µg/ml), separated by narrow intervals was evaluated for the test item to define as closely as possible the test concentration at which the replication index (RI) was reduced by approximately 60 %. This test concentration is used as the highest concentration to be evaluated. Two lower test concentrations were selected to cover a range of low (none) to maximum (60%) cytotoxicity. Negative and positive controls were in accordance with the draft guideline.

Results
Due to a steep toxicity curve a proper dose selection was complicated and, therefore two trials were required to select the proper concentrations for the analysis of micronuclei for each experiment. The highest test concentrations to be evaluated in experiment 1 were 53.15 µg/ml (cytotoxicity: 42 %) and 461.4 µg/ml (cytotoxicity: 66 %) in the absence and presence of metabolic activation, respectively. As a steep toxicity curve was noted for the test item, the next slightly higher concentration tested (59.05) induced 74 % cytotoxicity and was not suitable for evaluation. In experiment 2, the doses of 59.05 µg/ml and 810.0 µg/ml caused cytotoxicity of about 59 % and 61 %, respectively.
Treatment of cells with the test article in the absence of metabolic activation (experiment one and two) and in the presence of metabolic activation (experiment 1) resulted in frequencies of micronucleated binucleate cells (MNBN) that were similar to and not significantly different from those observed in concurrent solvent control cultures for all the concentrations tested. In experiment two with treatment of cells with the test article in the presence of metabolic activation resulted in frequencies of MNBN cells that were significantly higher compared to the solvent controls for all the concentrations tested. Thus, the most potent response (at the highest dose level) yielded 5.7% MNBN cells as compared to 0.5% MNBN cells in the solvent control.

Conclusion
2-chloro-6-ethylamino-4-nitrophenol caused chromosomal damage in the presence of metabolic activation, when treatment was commenced 48 hours following mitogen stimulation, as an increase in micronuclei formation was noted under these test conditions. No indications in micronuclei formation were observed 24 h following mitogen stimulation or for the test conditions in the absence of metabolic activation. Based on these results 2-

chloro-6-ethylamino-4-nitrophenol is evaluated as clastogenic and/or aneugenic in the presence of S9-mix in this *in vitro* assay.

Ref: 27

Comment on the *in vitro* studies
An *in vitro* SCE test in CHO cells has also been performed with 2-chloro-6-ethylamino-4-nitrophenol and had been presented in a former submission. As this study cannot be considered as valid due to marked limitations with regard to study design and reporting, the study is not presented here in detail. Result: negative/equivocal.

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian Erythrocyte Micronucleus Test

Guideline:	OECD 474 (1997)
Species/strain:	NMRI mice
Group size:	6 male mice/group
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Lot no:	9801090301
Purity:	99.9% (HPLC)
Dose level:	24 h: 437.5, 875 and 1750 mg/kg bw 48 h: 1750 mg/kg bw
Route:	Oral, gavage
Vehicle:	PEG 400
Sacrifice times:	24 h, and with the highest dose also at 48 h
GLP:	in compliance

2-chloro-6-ethylamino-4-nitrophenol was tested for its clastogenic/aneugenic potential in bone marrow cells of mice. Dose selection was based on findings in the pre-experiments for toxicity covering a range of 100 to 2000 mg/kg bw administered to two female and two male mice each using the identical treatment procedure. Femoral bone marrow was sampled from mice after sacrifice (24 hours after dosing) for all dose groups and additionally after 48 h for the high dose group. Bone marrow of the negative and concurrent positive control group animals was sampled 24 h after administration. Slides were prepared from the bone marrow preparations, stained with May-Grünwald/Giemsa and evaluated for the number of polychromatic erythrocytes (PCE) with micronuclei. At least 2000 PCEs per animal were analysed. In addition, the ratio between polychromatic and total erythrocytes per animal was determined. Five male animals per test group were evaluated as described above, except at the highest dose where only 4 animals survived. The animals of the high dose group were examined for acute toxic symptoms several times within the first 24 hours of treatment. Negative control groups received 10 ml/kg bw PEG 400 and concurrent positive control groups received 10 ml/kg bw cyclophosphamide (4 mg/ml) dissolved in deionised water.

Result
In the pre-test, 1 of 4 animals died within 1 h after administering a dose of 2000 mg/kg bw. At 1750 mg/kg bw, clinical signs as abdominal position, ruffled fur, eyelid closure and reduction of spontaneous activity were noted partially within the first 24 h, but no animal died. This dose was therefore selected as the highest test dose for the main experiment. No differences between males and females were noted in this pre-test. Therefore only male mice were used in the main study.
In the main study 2 animals died 1 h post treatment in the high dose group. Toxic signs like those described in the pre-experiment were noted in all dose groups, up to 24 hours after administration. In the highest dose group (1750 mg/kg bw) signs like reduction of spontaneous activity and ruffled fur were observed even 48 h after administration. The ratio of PCE/NCE did not reveal any treatment related effect. Hence, 2-chloro-6-ethylamino-4-nitrophenol tested at systemically toxic doses showed no clear cytotoxic effect in the bone

marrow. However, the urine of the treated animals had taken the colour of the test item indicating a good bioavailability of the test item after oral administration.

In comparison to the corresponding vehicle controls there was no statistically significant elevation in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. At the highest dose level (1750 mg/kg bw) after 24 hours treatment, the number of the micronucleated PCE's for animal no. 23 (7 per 2000 PCE's) was higher than the rest of the group and thereby resulting in 0.13% PCE's with micronuclei for the four animals compared to 0.060% for the vehicle control. 0.13% was not statistically significantly higher than the vehicle control and it was within historical control range.

Conclusion
It is concluded that under the test conditions reported in this study, 2-chloro-6-ethylamino-4-nitrophenol did not induce clastogenic activity as determined by the micronucleus test in the bone marrow cells of mice.

Ref.: 28

Comet Assay *in vivo*

Guideline:	/ (according to an internationally accepted protocol for the Comet assay *in vivo*)
Species/strain:	Wistar HanIbm: WIST (SPF) rats
Group size:	6 males per dose group
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Batch:	9801090301
Purity:	99.9 area % (HPLC,)
Dose levels:	82.5 and 250 mg/kg bw
Route:	Oral, gavage
Treatment:	Each animal was dosed twice 19 and 3 h before sacrifice except for the positive control group (3 h only)
Vehicle:	PEG 400
GLP:	In compliance

The potential genotoxicity of 2-chloro-6-ethylamino-4-nitrophenol was assessed in the Comet assay in liver, small intestine and urinary bladder epithelium cells after *in vivo* treatment of male rats. The test item was dissolved in PEG 400 and administered via gavage (dosing volume 10 ml/kg bw) twice to 6 male Wistar rats at doses of 0, 82.5 and 250 mg/kg bw 19 and 3 hours before sacrifice. The positive control group received 25 mg/kg bw methylmethane sulfonate in a 0.9 % NaCl solution once via gavage 3 h before sacrifice.
A number of pre-experiments were conducted using 2 animals per sex and group to identify the maximum tolerated dose. Mortality occurred at 500 and 1000 mg/kg bw. test compound, signs of systemic toxicity appeared in animals receiving 125 and 250 mg/kg bw.
The doses used in the main study were selected based on the observed clinical signs in these pre-tests (reduction of spontaneous activity, ruffled fur and discoloured urine) that demonstrated systemic toxicity and bioavailability of the test item.
Intact cells of the different organs were isolated by in situ perfusion of the liver, by enzymatic digestion of urinary bladder epithelium and by tissue mincing of the small intestine of 5 treated animals per group. Subsequently, cells were mixed with agarose, lysed and subjected to the electrophoresis under alkaline conditions.
After staining the slides with ethidium bromide, 50 cells per slide and two slides per animal (100 cells in total) were evaluated for the occurrence of DNA damage expressed as tail length, Olive tail moment and % tail DNA. The viability of hepatocytes and bladder epithelium cells was determined directly after the cell isolation by using the trypan blue

exclusion method. Due to the mincing procedure, viability of small intestinal cells could not be determined.

Results
The pre-experiments demonstrated systemic toxicity to 2-chloro-6-ethylamino-4-nitrophenol up to and including 1000 mg/kg. In the main study, discoloured urine after administration of all doses as well as diarrhoea, ruffled fur and reduced spontaneous activity demonstrated systemic availability of the test item.
The viability of the hepatocytes and bladder epithelium cells was not substantially affected by the in vivo treatment with the test substance. The viability of the small intestine cells could not be analysed due to the mincing procedure.
None of the tested dose levels caused an increase in DNA damage in the hepatocytes, the bladder epithelium, or the small intestinal epithelial cells of the treated animals as compared to the corresponding vehicle control in any of the evaluated parameters (Olive Tail Moment, Tail Length, and Tail % DNA).
In vivo treatment with the positive control MMS revealed distinct and significant increases in the Olive Tail Moment, the Tail % DNA, and the Tail Length.

Conclusion
It is concluded that under the test conditions reported in this study, 2-chloro-6-ethylamino-4-nitrophenol did not induce DNA damage in the Comet assay performed in cells isolated from the liver, bladder or the small intestine of rats.

Ref: 29, 30

Comment
An *in vivo/in vitro* UDS test in Sprague-Dawley CD rats has also been performed with 2-Chloro-6-ethylamino-4-nitrophenol in 1995. As this test had several limitations with regard to the study design, the study is not reported here in detail. The result was negative.

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

Guideline:	OECD 414
Species/strain:	Rat, strain Wistar Crl:Wi /Br
Group size:	20 females per dose group
Test substance:	2-chloro-6-ethylamino-4-nitrophenol
Batch:	AR 917
Purity:	99.7%
Dose levels:	10, 30 and 90 mg/kg bw in 0.5% Carboxymethylcellulose (CMC) in water
Route:	Oral, gavage
Exposure:	Once daily from day 5 to day 15 of pregnancy
GLP:	In compliance

2-Chloro-6-ethylamino-4-nitrophenol in a constant volume of 10 ml/kg bw (0.5% CMC), was administered once daily by oral gavage to groups of 20 pregnant rats (successful

mating was verified by sperms in vaginal smear) at doses of 10, 30 and 90 mg/kg bw from day 5 to day 15 of gestation. A control group received the vehicle only.
Animals were observed at least once daily for clinical signs during the entire treatment period. Body weights were recorded at days 0, 5, 10, 15 and 20 of gestation. Food consumption was measured for the intervals day 0-5, 5-15 and 15-20 and calculated for the entire study period.
On day 20 of gestation, all mated females were sacrificed and a complete necropsy and a macroscopic examination of the organs were carried out. The ovaries and the intact uterus were removed and the presence of resorption sites and foetuses were examined. The numbers of implantation sites and of corpora lutea were also determined. Each live foetus was weighed, sexed and examined for gross external malformations. Also a skeletal (about 2/3 of the foetuses) and a visceral (about 1/3 of the foetuses) examination of the foetuses was performed and placenta and uterus were weighed.

Results
There were no signs of maternal toxicity either during the observation period or at necropsy. The body weight, body weight gain and the food consumption were not affected by the treatment with 2-chloro-6-ethylamino-4-nitrophenol. Gross necropsy revealed no treatment related effects. The only treatment-related effect was discolouration of the urine in the treatment groups.
There were no treatment related effects with regard to litter size, foetal mortality, foetal body weight and sex ratio. The placenta weights, the number of corpora lutea, and the number of implantations were similar to control values in all treated groups.
The gross necropsy and the skeletal and visceral examination of the foetuses revealed no treatment related findings. Neither a statistically significant difference nor a dose-dependent increase in any malformation was noted. The observed variations represented common findings for the rat strain used and were within the spontaneous variation range and/or revealed no dose-response relation.

Conclusion
2-Chloro-6-ethylamino-4-nitrophenol revealed neither maternal toxic effects nor did it induce embryotoxicity or teratogenicity in female rats. Based on the results of the study the NOAEL for both maternal and developmental toxicity was a dose of 90 mg/kg bw administered during organogenesis.

Ref.: 31

Comment
For hazard identification, the highest dose applied in teratogenicity studies should lead to maternal and/or developmental toxicity

3.3.9. Toxicokinetics

Guideline:	/
Species/strain:	Rat, strain Sprague Dawley Him:OFA (SPF)
Group size:	3 per sex and dose
Test substance:	^{14}C-2-chloro-6-ethylamino-4-nitrophenol (ring-labelled)
Batch:	109F9206
Purity:	> 98% (Radiochemical, HPLC), specific activity 12.0 mCi/mmol
Route of application:	Experiments A, B, C: dermal Experiments D, E: oral
Dose levels:	Exp. A: Formulation without H_2O_2: 2%; 1.76 mg/cm^2 Exp. B: Formulation with H_2O_2; 2%; 2.14 mg/cm^2 Exp. C: Solution in water/DMSO (3:7); 6.66%; 2.31 mg/cm^2 Exp. D: Solution in water/DMSO (4:6); 2%; 102.4 mg/kg bw Exp. E: Solution in water/DMSO (4:6); 2%; 102.2 mg/kg bw
Exposure:	Exp. A, B, C: Single dermal application (30 min) Exp. D, E: Single oral administration (gavage)

GLP: In compliance

Dermal application (experiments A, B and C)
^{14}C-2-Chloro-6-ethylamino-4-nitrophenol was applied dermal to groups of 3 male and 3 female rats. The application area was 9 cm² and the test substance was applied at concentrations of 6.66 % in solution (water/DMSO 3:7, experiment C) and of 2% in formulations without (experiment A) and with hydrogen peroxide (experiment B) for 30 min. The mean amount of the applied dyestuff corresponded to 1.76, 2.14 and 2.31 mg/cm², respectively.
For termination of exposure, the test substance was scraped off and the skin rinsed with a shampoo formulation followed by water. Rinsing solutions were collected. During the exposure time, animals were constrained to avoid licking. After rinsing, the area was covered with gauze fixed by adhesive tape and an additional air permeable plastic cone to further prevent licking of the treated area during the 72 h in the metabolism cages.

Oral administration (experiments D and E)
102.4 mg/kg bw (exp. D) and 102.2 mg/kg bw (exp. E) ^{14}C-2-chloro-6-ethylamino-4-nitrophenol were administered as a 2% solution in water/DMSO 4:6 by gavage to two groups of 3 male and 3 female rats each which were starved for 16 hours before treatment. In experiment D, animals were placed in metabolism cages for 72 h. In experiment E, blood was taken at several time points within 24 h after administration.
Urine and faeces were collected daily (0-24, 24-48 and 48-72 h after administration) from the metabolic cages.
Animals were killed 72 hours (experiments A, B, C, and D) and 24 hours (experiment E) after the application and the application sites (experiments A, B and C) and various organs were taken and analysed for radioactivity. The radioactivity in the remaining carcass after skin removal (experiments A, B, C and D) or removal the gastro-intestinal tract (experiment D) was determined.

Results
Total recovery of the applied radioactivity for the individual animals in dermal and oral experiments ranged from 95.85 to 98.52% and from 91.9 to 94.1%, respectively.

Dermal application
Seventy two hours after application, the amount of radioactivity remaining in the skin was less than 1 % for all three experiments. The highest value (0.5 % of the applied dose) was noted for the solution in DMSO/water (experiment C). For the formulation without H_2O_2 (experiment A) and with H_2O_2 (experiment B), the figures were 0.18 and 0.47 %, respectively. The majority of the dyestuff (94.7 to 96.9%) was removed by the rinsing water.

0.14%, 0.10% and 2.83% of the applied doses were eliminated via urine and faeces in 72 h in experiment A, B and C, respectively. The lowest absorption rate was obtained for the formulation containing H_2O_2. Radioactivity was mainly excreted via urine (85–88%) and elimination was fast, since 90% of the total amount eliminated was excreted within the first 24 hours. The radioactivity remaining in the carcass (0.0021 to 0.0028% of the administered and 1.0 to 2.1% of the adsorbed dose) was low 72 hours after administration. Residues in organs were mostly below the detection limit, with highest concentrations noted for kidneys and thyroids.
A cutaneous absorption rate of 0.14%, equal to 2.51 µg/cm² for the formulation without H_2O_2, 0.10% equal to 2.16 µg/cm² for the formulation with H_2O_2 and 2.83% equal to 66.31 µg/cm² for the 2-chloro-6-ethylamino-4-nitrophenol in water/DMSO were obtained. The data obtained in experiment A and B showed a slightly higher absorption rate and a higher bioavailability of 2-chloro-6-ethylamino-4-nitrophenol for males than for females, although most of the parameters like urine, faeces or carcass/organ residues revealed no statistically significant differences. The opposite trend was noted in experiment C.

Oral administration
After oral administration, 2-chloro-6-ethylamino-4-nitrophenol was mainly eliminated via urine (62% of the applied dose within 72 h) and to a minor extent via the faeces (31%). Elimination was fast and the substance was mainly excreted within the first 24 hours (77%). The radioactivity found in tissues 72 h after administration was generally low (less than 0.03% of the applied dose per g organ), with higher values noted in kidneys, adrenals, liver and thyroids compared to other organs.
The blood level (experiment E) reached a peak at 35 min after application and declined with an initial half-life of 1 h.
After oral application, a trend towards higher residual levels was noted for several organs in males as compared to females. The finding is consistent with the blood levels obtained in experiment E, in which higher blood levels were also noted for males.

Conclusion
2-Chloro-6-ethylamino-4-nitrophenol given orally to rats is quickly absorbed and practically excreted within 72 h, with the majority eliminated within 24 h after application. Excretion takes place predominantly (62%) via urine and to a minor extent via faeces, demonstrating bio-availability after oral application.
After dermal application only minor amounts (max. 66.31 µg/cm², equal to 2.8%) are absorbed through the skin and become systemically available. Significantly lower figures are obtained if hair dye formulations as vehicle are used (2.16 µg/cm² and 2.51 µg/cm² for formulations with and without hydrogen peroxide). The observed excretion pattern is very similar to the one observed for the oral route, with the majority excreted via urine within 24 hours after administration.
Low tissue residue levels were noted for both routes of exposure. The remaining radioactivity in the skin after dermal application was also low (less than 1% of the applied dose).
There were no significant and consistent sex differences in the absorption, tissue distribution and excretion pattern.

Ref.: 33

Comment
In this study, a concentration of only 2% was used whereas the maximum intended concentration is 3%. Therefore, for the calculation of the Margin of Safety, a value of 2.51 µg/cm² was extrapolated (times 1.5 = 3.77 µg/cm²).

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-chloro-6-ethylamino-4-nitrophenol)
(non-oxidative)

Maximum absorption through the skin	**A (µg/cm²) µg/cm²**	**=**	**3.77**
Skin Area surface	**SAS (cm²)**	**=**	**700 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**2.64 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.04 mg/kg**
No observed effect level (mg/kg) (90-day, oral, rat)	**NOEL**	**=**	**10 mg/kg**
Margin of Safety	**NOEL / SED**	**=**	**250**

3.3.14. Discussion

Physico-chemical properties
2-Chloro-6-ethylamino-4-nitrophenol is used as an ingredient in oxidative hair dye formulations at a maximum on-head concentration of 1.5 %. It is also used as a hair colouring agent in semi-permanent hair dye formulations at a maximum on head concentration of 3 %.
No documentation is provided for the characterisation and composition of various batches of 2-Chloro-6-ethylamino-4-nitrophenol.
2-Chloro-6-ethylamino-4-nitrophenol is a secondary amine, and thus it is prone to nitrosation. Nitrosamine content in 2-Chloro-6-ethylamino-4-nitrophenol is not reported. It should not be used in combination with nitrosating agents.
Stability of 2-Chloro-6-ethylamino-4-nitrophenol in marketed products is not provided.

General toxicity
The acute median lethal oral doses (LD_{50}) of 2-chloro-6-ethylamino-4-nitrophenol in rats were 1461 mg/kg bw for females, 2026 mg/kg bw for males and 1728 mg/kg bw for both sexes. The acute median lethal dermal dose (LD_{50}) of 2-chloro-6-ethylamino-4-nitrophenol was > 2000 mg/kg bw in both sexes of rats. Due to statistically significantly increased liver weight in male rats observed at 30 and 90 mg/kg bw after the 90 days exposure a NOAEL of 10 mg/kg bw was deduced. 2-Chloro-6-ethylamino-4-nitrophenol revealed neither maternal toxic effects nor did it induce embryotoxicity or teratogenicity in female rats on the highest exposure level used (90 mg/kg bw). Based on this the NOAEL for both maternal and developmental toxicity was a dose of 90 mg/kg bw administered during organogenesis.

Toxico-kinetics
2-Chloro-6-ethylamino-4-nitrophenol given orally to rats is quickly absorbed and practically completely excreted within 72 h, with the majority eliminated within 24 h after application. Excretion takes place predominantly (62%) via urine. After dermal administration (vehicle/water/DMSO) up to 66.31 µg/cm², equal to 2.8% are absorbed through the skin and become systemically available. Lower figures are obtained if hair dye formulations are used (2.16 µg/cm² and 2.51 µg/cm² for formulations without and with hydrogen peroxide).

Irritation / sensitisation
ROT CO applied undiluted in solid form or as a 3 % solution in propylene glycol, was not irritating to intact or abraded skin of rabbits after 4 hours occlusion. Undiluted *ROT CO*

(solid form) was irritating to rabbits eyes. Dissolved in propylene glycol at 3 %, it showed transient irritation in a rabbit eye.
According to a LLNA, it revealed moderate skin sensitising properties when applied in DMSO.

Dermal absorption
Under the described test conditions (pig skin *in vitro*, 30 minutes exposure, 72 hours duration, only non-oxidative conditions), a maximum skin penetration rate (A_{max}) of 2.1µg/cm² (0.069 % of the applied dose) of *ROT CO (WR23192)* was obtained by summing up the amounts for receptor fluid and for the lower skin compartment (upper dermis).
The number of replicates used in this study (1 donor, 5 chambers) was not according to the SCCP Notes of Guidance. The test was only performed under non-oxidative conditions. Therefore, the study cannot be accepted.

In an *in vivo* toxicokinetics study in rats, the percutaneous absorption of 2-chloro-6-ethylamino-4-nitrophenol was determined as 0.14 % (2.51 µg/cm²) and as 0.1 % (2.16 µg/cm²) of the amount of radioactivity applied dermally as part of non-oxidative and oxidative (in the presence of hydrogen peroxide) hair dye formulations, respectively. In this study a concentration of only 2 % was used whereas the maximum intended concentration is 3 %. Therefore for the calculation of the Margin of Safety, a value of 2.51 µg/cm^2 was extrapolated (times 1.5 = 3.77 µg/cm^2).

Mutagenicity / genotoxicity
Overall, 2-chloro-6-ethylamino-4-nitrophenol has been investigated for the induction of the three genetic endpoints: gene mutations, structural chromosome aberrations and aneuploidy.
2-chloro-6-ethylamino-4-nitrophenol induced gene mutations in one of two bacteria mutations tests. A clear effect was seen without S9-mix and a marginal effect with S9-mix. In the mouse lymphoma assay with S9-mix there was an increase in large colonies indicating induction of point mutations. Clastogenic effect was observed in the micronucleus test in human lymphocytes and the mouse lymphoma assay (small colonies) with S9-mix in both assays.
There were no indications for genotoxic effects of 2-chloro-6-ethylamino-4-nitrophenol *in vivo,* based on results from a micronucleus test in the bone marrow of mice and a Comet assay on cells isolated from the liver, bladder or the small intestines of rats. A rat *in vivo/in vitro* UDS test of limited validity was also negative.
Consequently, 2-chloro-6-ethylamino-4-nitrophenol is considered to have no a relevant mutagenic potential *in vivo*. Additional tests are not necessary.
To reach a definitive conclusion, appropriate tests with 2-chloro-6-ethylamino-4-nitrophenol in combination with hydrogen peroxide should be provided.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the use of 2-chloro-6-ethylamino-4-nitrophenol, at a maximum on-head concentration of 1.5% in oxidative hair dye formulations and at a maximum on-head concentration of 3.0% in non-oxidative hair dye formulations, does not pose a risk to the health of the consumer, apart from its sensitising potential.

However, no documentation is provided for the characterisation and composition of various batches of 2-Chloro-6-ethylamino-4-nitrophenol.

2-Chloro-6-ethylamino-4-nitrophenol is a secondary amine, and thus it is prone to nitrosation. It should not be used in combination with nitrosating substances.
The nitrosamine content should be < 50 ppb. The nitrosamine content in 2-Chloro-6-ethylamino-4-nitrophenol was not reported.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP/SCCP opinions and in accordance with its Notes of Guidance.

5. MINORITY OPINION

Not applicable

6. REFERENCES

1. Lenz, Dr.; Report partition coefficient Rot CO; *BIOCHEM*; 1992
2. Merritt, M.; Determination of the particle size; *LCG BIOSCIENCE*; 2001
3. Lenz, Dr.; Report melting point Rot CO; *BIOCHEM*; 1992
4. Lenz, Dr.; Report boiling point Rot CO; *BIOCHEM*; 1992
5. Lenz, Dr.; Report relative density Rot CO; *BIOCHEM*; 1992
6. Lenz, Dr.; Report vapour pressure curve Rot CO; *BIOCHEM*; 1992
7. Lenz, Dr.; Report surface tension (aqueous solution) Rot CO; *BIOCHEM*; 1992
8. Lenz, Dr.; Report water solubility Rot CO; *BIOCHEM*; 1992
9. Lenz, Dr.; Report inflammability (solids) Rot CO; *BIOCHEM*; 1992
10. Mücke, O.; Bericht über die Prüfung des Stoffes 2-Chlor-6-ethylamino-4-nitrophenol ("Rot CO") auf explosive Eigenschaften; *BAM*; 1993
11. Lenz, Dr.; Report auto-flammability (solids) Rot CO; *BIOCHEM*; 1992
12. Lenz, Dr.; Report flame supporting properties Rot CO; *BIOCHEM*; 1992
13. Kemper, F. H., Vahlensieck, U., Hambrügge, M.; SCC opinion concerning: Ethylchloroorange Colipa N°.: B 89; *SCC*; 1996
14. Chibanguza, G.; Akute orale Toxizität an Ratten mit Rot CO in Anlehnung an die OECD-Richtlinien; *IBR*; 1988
15. Klein, W.; Acute dermal toxicity of "Rot CO" in rats (Limit-test); *SEIBERSDORF*; 1992
16. Chibanguza, G.; Prüfung von Rot CO im Hautreiztest am Kaninchen; *IBR*; 1988
17. Chibanguza, G.; Prüfung von COS 552 Rot CO im Hautreiztest am Kaninchen; *IBR*; 1988
18. Chibanguza, G.; Prüfung von Rot CO im Augenreiztest am Kaninchen; *IBR*; 1988
19. Chibanguza, G.; Prüfung von COS 552 Rot CO im Augenreiztest am Kaninchen; *IBR*; 1988
20. Ravel, G.; 2-Chloro-6-ethylamino-4-nitrophenol WR 23192 - Local lymph node assay; *MDS PHARMA SERVICES*; 2004
21. Wyss, C.; Cutaneous Absorption of 2-Chloro-6-ethylamino-4-nitrophenol (= WR23192) in "basic formulation for ringtest with 3 % Rot CO" (derived from a typical hair dye formulation) through pig skin in vitro; *COSMITAL SA*; 2004

22. Simon, G. A.; Maibach, H. I.; The pig as an experimental animal model of percutaneous permeation in man: Qualitative and quantitative observations - An overview; *SKIN PHARMACOL. PHYSIOL.*; 13, *229-234*; 2000
23. Korn, W.-D.; 3-month oral toxicity study with "ROT CO" (Cos. 552) in rats (followed by a 4-week recovery period); *IBR*; 1989
24. Grötsch, W.; Final report of the study to determine the ability of the test compound COS 552 to induce mutations in the Ames Salmonella/Microsome plate incorporation assay; *L+S GmbH*; 1993
25. Sokolowski, A.; Salmonella Typhimurium reverse mutation assay with 2-Chloro-6-ethylamino-4-nitrophenol (WR 23192); *RCC-CCR*; 2005
26. Ballantyne, M.; 2-Chloro-6-ethylamino-4-nitrophenol (WR23192): Mutation at the Thymidine Kinase (tk) locus of mouse lymphoma L5178Y cells (MLA) using the microtitre fluctuation technique; *COVANCE*; 2005
27. Whitwell, J.; WR 23192: Induction of micronuclei in cultured human peripheral blood lymphocytes; *COVANCE*; 2004
28. Honarvar, N.; Micronucleus assay in bone marrow cells of the mouse with 2-Chloro-6-ethylamino-4-nitrophenol (WR 23192); *RCC-CCR*; 2005
29. Hartmann, A.; Agurell, E.; Beevers, C.; Brendler-Schwaab, S.; Burlinson, B.; Clay, P.; Collins, A.; Smith, A.; Speit, G.; Thybaud, V.; Tice, R. R.; Recommendations for conducting the in vivo alkaline Comet assay; *MUTAGENESIS;* 18, *45-51*; 2003
30. In vivo alkaline single cell gel electrophoresis analysis of various rat organs with 2-Chloro-6-(ethylamino)-4-nitrophenol (WR 23192); *RCC-CCR; 2005*
31. Korn, W.-D.; Teratogenicity study with "Rot CO (Cos 552)" in Wistar rats; *IBR*; 1989
32. Kennedy, K. J.; ADME: A-B Permeability - Study of 2-Chloro-6-ethylamino-4-nitrophenol; *CEREP*; 2004
33. Reindl, E.; Toxicokinetics of "Rot CO"; *SEIBERSDORF*; 1990
34. König, P.; Data base search for references for B089 - 2-Chloro-6-ethylamino-4-nitrophenol: 131657-78-8; *WELLA AG*; 2005