The oil companies' European association for Environment, Health and Safety in refining and distribution

concawe

report no. 12/12

Use of the modified Ames test as an indicator of the carcinogenicity of residual aromatic extracts

ISBN 978-2-87567-014-4

Use of the modified Ames test as an indicator of the carcinogenicity of residual aromatic extracts

Prepared for the CONCAWE Health Management Group by its Special Task Force H/Tox Subgroup:

P. Boogaard
A. Hedelin
A. Riley
E. Rushton
M. Vaissiere

G. Minsavage (Technical Coordinator)
A. Rohde (Technical Coordinator)
W. Dalbey (Consultant)

Brussels
December 2012

ABSTRACT

Existing data demonstrate that residual aromatic extracts (RAEs) can be either carcinogenic or non-carcinogenic. CONCAWE had previously concluded that "Although limited data available indicate that some RAEs are weakly carcinogenic, it is not possible to provide a general recommendation. Classify on a case-by-case basis" (CONCAWE 2005) [11]. Therefore CONCAWE's Health/Toxicology Subgroup (H/TSG) has developed a proposal for the use of the modified Ames test as a short-term predictive screening tool for decisions on the classification of RAEs for carcinogenicity.

The relationship between RAE chemistry and carcinogenic potential is not as well understood as it is for some other categories of substances, e.g. Other Lubricant Base Oils (OLBO). However, a correlation has been found between the results of the skin carcinogenicity bioassay and the mutagenicity index (MI) obtained from the modified Ames test. Data supporting this correlation are summarised in this report. The H/TSG confirmed that the modified Ames test can be used as a predictive screening tool and that a cut-off value can be established to make a distinction between carcinogenic and non-carcinogenic products. RAEs with a MI $\geq$ 0.4 demonstrated carcinogenic potential upon dermal application to mouse skin with chronic exposure. RAEs with a MI < 0.4 did not demonstrate a carcinogenic potential.

To justify the use of the modified Ames test with RAEs, additional analysis of the repeatability of the test with RAEs was required. With this objective, CONCAWE sponsored a round robin study with different samples of RAEs from member companies, at three different laboratories. The repeatability demonstrated in the round robin study with RAEs support the proposed use of the modified Ames test.

As part of the tools available for use by member companies, the H/TSG proposed a standard operating procedure (SOP) (included as an Appendix to this report) on the conduct of the modified Ames test with RAEs. The H/TSG also prepared two special Oil Industry Notes (OINs) for use in cancer hazard classification. One OIN based on Dangerous Substances Directive (DSD) (OIN 9) and one based on Classification, Labelling and Packaging (CLP) regulation (OIN 10) have been adopted.

KEYWORDS

Aromatic extract, Bright stock extract, Carcinogenicity, Dermal, Modified Ames, Mutagenicity, Mutagenicity index, Process oil, Residual aromatic extract

INTERNET

This report is available as an Adobe pdf file on the CONCAWE website (www.concawe.org).

CONTENTS

SUMMARY

Background
This category of petroleum substances (Residual Aromatic Extracts, RAEs) covers two CASRNs/EINECS[1] describing residual aromatic substances used as feedstocks for synthesis of other petroleum substances or as discrete substances themselves. **Appendix 1** provides the definition of the two substances.

Following the procedure for classification and labelling of petroleum substances according to the European Union's (EU) DSD, CONCAWE (2005) [11] concluded that "Although limited data available indicate that some RAEs are weakly carcinogenic, it is not possible to provide a general recommendation. Classify on a case-by-case basis." With the implementation of the REACH[2] and CLP[3] regulations, this statement needed to be expanded to provide more complete guidance. Therefore the Health Toxicology Subgroup (H/TSG) has developed a new criterion for the classification of RAE as carcinogenic, based on the assessment of available data.

Tests Reviewed or Conducted by the CONCAWE Health Toxicology Subgroup
Existing data demonstrate that individual RAEs may be carcinogenic or non-carcinogenic. A conservative method was applied, using the category approach, during the preparation of the REACH registration dossiers. That is, the data showing the greatest hazard for a given endpoint determined the resultant hazard classification for the category. If the hazard of a particular substance within that category was demonstrated to be different from that default hazard, it was determined that a discriminator could be used to distinguish that substance. During the REACH registration process, multiple skin carcinogenicity bioassay and modified Ames test reports became available. For some samples tested, the data supported a Category 3 (DSD) or Category 2 (CLP) classification while data for other samples warranted no classification.

However, if a 'worst case' approach were followed with no further consideration of the data, all RAE substances would need to be classified as Category 3 (DSD) or Category 2 (CLP).

The relationship between RAE chemistry and carcinogenic potential is not as well understood as it is for some other categories, e.g. Other Lubricant Base Oils (OLBO). Almost all skin-painting studies on RAEs have been performed in parallel with a modified Ames test. A correlation has been found for RAEs between the results of the skin carcinogenicity bioassay and the mutagenicity index (MI) obtained from the modified Ames test, but that correlation appeared to differ from the correlation for OLBOs [10]. In order to evaluate the potential use of the modified Ames test to assess potential carcinogenicity of RAEs, data were compiled and reviewed to determine whether a cut-off could be established using the MI as an indicator of potential carcinogenicity of a given sample of RAE.

Additional validation of the use of the modified Ames assay with RAEs was needed since the results from the studies were close to the biological variability expected with a null result. (MI < 0.4) Therefore, CONCAWE sponsored a round robin study of modified Ames tests on seven samples of RAEs at three different laboratories. The

[1] EINECS: European Inventory of Existing Commercial Chemical Substance
[2] REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals (EU 2006)
[3] CLP : Classification, Labelling and Packaging (EU 2009)

goal was to evaluate the intra- and inter-laboratory repeatability of results from the modified Ames test.

CONCAWE H/TSG has proposed a Standard Operating Procedure (SOP) on the conduct of the potential utility modified Ames test with RAEs. This SOP is included as an Appendix 6 to this report for possible use by member companies. There is no recommendation on which laboratory to use.

Conclusions

Based on the reliability of the modified Ames test and the results from skin-painting studies, CONCAWE H/TSG has confirmed that the modified Ames test can be used as a screening tool for assessing the potential carcinogenicity activity of RAEs. A cut-off value was determined to distinguish between carcinogenic and non-carcinogenic RAE samples. Data demonstrate that RAEs with an MI $\geq$ 0.4 demonstrated potential carcinogenic activity to mouse skin following chronic exposure. RAEs with a MI < 0.4 did not demonstrate a carcinogenic potential.

The results from the round robin study with RAEs support the use of the modified Ames test as a predictive screening tool for potential carcinogenic activity of RAEs.

CONCAWE has prepared two classification notes: one Oil Industry Note (OIN) based on the DSD regulation (OIN 9) and one based on the CLP regulation (OIN 10).

1. INTRODUCTION

1.1. DEFINITION OF RESIDUAL AROMATIC EXTRACTS

RAEs are highly viscous or semi-solid petroleum hydrocarbon streams, derived as the solvent extract of residual oils from the vacuum distillation of atmospheric residues. They consist predominantly of aromatic hydrocarbons having carbon numbers higher than C25. In the EU, the chemical name for RAEs is either 'Extracts (petroleum), residual oil solvent' or 'Residues (petroleum), vacuum' described by two CAS registry number (RN) (or EC numbers), namely 64742-10-5 (265-110-5) and 91995-70-9 (295-332-8) respectively. Synonyms include bright stock extract, aromatic process oil, process oil, and aromatic extract. Additional details are in **Appendix 1**.

1.2. MANUFACTURE AND USES

RAEs are produced during the refining of lubricating oil basestocks and waxes. The residue (residuum) of atmospheric distillation of crude oil is distilled under vacuum to produce distillate and residual lubricating oil basestocks. The untreated lubricating oil basestocks contain undesirable components that negatively impact lubricant performances, i.e., colour, odour, stability and/or viscosity, and therefore must be removed. These undesirable components include polycyclic aromatic compounds (PACs) and aromatic compounds containing sulfur, nitrogen, and oxygen as heteroatoms.

One way in which these undesirable components can be removed is to incorporate a solvent extraction step. In the production of RAE, the residuum from vacuum distillation is extracted with liquid propane to remove particulates, resins, and asphaltenes. In this process, the resins, asphaltenes, and particulates precipitate and the propane/oil stream is then stripped of the propane. The very viscous stream that results from this process is referred to as deasphalted oil (DAO). The DAO then undergoes a second solvent extraction process, for example with furfural, to remove aromatic compounds. The solvent is removed from this extract, leaving the RAE.

Historical international values on various physical and chemical properties for RAEs were summarized previously [9]. The viscosity of RAEs increases with increasing boiling range [7,3]. Note that the IP346 method (IP, 1985), used as a screening assay for the potential dermal carcinogenicity of lubricating base oils based on measurement of solvent extractables is not considered appropriate for RAEs [10,32].

RAEs are used mainly in industrial and professional applications. These uses of RAEs include coatings, metalworking, release agents or binders, agrochemicals, road construction, rubber production and processing, lubricants, polymer processing and as a functional fluid. Consumer uses are limited to lubricants and coatings applications. Other uses are not recommended unless an assessment is completed, prior to commencement of that use, which demonstrates that the use will be controlled. Such additional assessment is the responsibility of the individual registrant

1.3. REGULATORY BACKGROUND FOR CLASSIFICATION OF RAES FOR CARCINOGENICITY AND GOALS OF THIS REVIEW

Under REACH it is necessary for manufacturers to determine the hazard classification of substances for a range of hazard endpoints, including the potential of the chemical to cause carcinogenic effects. The EU criteria used for assessing carcinogenic hazard under DSD and CLP are reproduced in **Appendix 2**, with key pieces of text in bold text.

The definitive test for dermal carcinogenic potential is the skin-painting assay in mice. The modified Ames test has been shown to be a useful predictive screening test that correlates with skin-painting data to assess the potential carcinogenicity of OLBOs, but not for any other category of petroleum substances.

The goal of the work summarized in this report was to evaluate the correlation between the results of the skin carcinogenicity bioassay and the MI obtained from the modified Ames test for RAEs. That evaluation included the following:

- Determination of whether a cut-off could be established for the MI as an indicator of potential carcinogenicity of a given sample of RAE (Appendix 4),
- A round robin study of modified Ames tests on seven samples of RAEs at three different laboratories to assess the intra- and inter-laboratory variability of data in the test (Appendix 5),
- Development of a SOP on the conduct of the modified Ames test with RAEs (Appendix 6), and
- Development of special OINs for use in cancer classification of RAEs.

2. OVERVIEW OF RAE HAZARD DATA

2.1. EXPERIMENTAL DATA ON CARCINOGENICITY OF RAES: IN VIVO STUDIES

Due to the physical nature and expected uses of RAEs (see Section 1.2), the skin is expected to be the primary route of human exposure under foreseen circumstances of use. For this reason, *in vivo* experimental studies on potential carcinogenicity have been performed using dermal application of RAEs. In general terms, numerous studies have shown that the mutagenic and carcinogenic potential of heavy petroleum streams correlates with the presence of PACs [35,13,5]. The results of dermal carcinogenicity of samples of RAE range from non-carcinogenic to positive findings in some tests [4,6,14,18,19,20,34].

The relationship between RAE chemistry and carcinogenic potential is not as well understood as it is for some other categories of petroleum substances, e.g. OLBOs. However, one contributing factor is the presence of potentially carcinogenic PACs. Solvent extraction of the vacuum residue will result in the extraction and concentration of aromatic molecules including PACs into the extract fraction ie. the RAE. Thus, the carcinogenic activity of an RAE may be greater than a vacuum residue from which it is derived. However, because the relationship between RAE chemistry and carcinogenic potential is not well understood, other mechanisms of carcinogenicity might also be operative.

In the typical skin-painting assay in mice, samples of the test substance are applied 2-3 times per week to the shaved dorsal skin for an extended time up to the lifetime of the animals. The primary endpoints in these studies are the appearance of papillomas or carcinomas during the biophase of the study and histological confirmation of the tumours (or lack thereof) after necropsy of the animals.

In the evaluation of results from mouse skin-painting studies, it is important to consider the background incidence of spontaneously occurring skin tumours, the latency period for the development of tumours, and the possible influence of dermal irritation on the overall result. Comments on these aspects are as follows:

- **Spontaneous incidence:** Oil industry experience with the testing of petroleum hydrocarbons in mouse skin painting studies suggests that a dermal tumour incidence of more than 4% is suggestive of a compound related carcinogenic response (i.e., 2 or more tumour bearing animals out of 50 animals tested can be considered to be a substance-related carcinogenic response). It is recognised that a 4% tumour incidence is a conservative threshold that might not reach statistical significance in any given study [5,8]. Additional discussion on this point is included in **Appendix 4**.
- **The dermal tumours** used to define a tumour-bearing animal (TBA) are dermal papillomas (benign) and carcinomas (malignant).
- **Tumour latency period:** In general, dermal tumours that develop following exposure to RAEs did not do so until between 50 and 78 weeks.

Dermal irritation: Based on information available in **Appendix 4**, with the exception of sample CRU 86518 which was reported to have provoked severe skin irritation, samples of RAEs cause no more than mild skin irritation. This irritation is not considered to be a significant confounding factor and insufficient for classification.

Eleven samples of RAEs have been tested using a mouse skin-painting model with either a 78-week or 104-week protocol and also in the modified Ames test. Dosing regimens varied in the skin-painting assays, involving twice weekly dosing with 37.5 µl, 50 µl or 50 mg of each RAE. In some studies the RAE samples were warmed to facilitate dermal application. Results of studies on the 11 samples are summarised **Appendix 4**.

2.2. GENETIC TOXICITY *IN VITRO* AS AN INDICATOR OF POTENTIAL CARCINOGENICITY

The Ames test was developed in the 1970s for assessment of the mutagenic activity of chemicals and for use as a tool to estimate their carcinogenic potential (Ames 1975). In this assay, multiple strains of histidine-dependent bacteria are exposed to the chemical with and without metabolic activation. Those bacteria with a mutation in DNA, which makes them histidine-independent, subsequently form colonies when plated on a growth medium that is deficient in histidine. These bacteria have reverted to the wild-type state. The numbers of revertant colonies that are able to grow in this medium are then counted.

When the Ames test was first applied to petroleum substances, two problems arose. First, the original Ames test was not controlled tightly enough to be quantitative. Second, the PACs in the insoluble oil samples did not become biologically active even in the presence of metabolic activation systems. The modified Ames assay was developed to overcome these obstacles and provide an assay designed for use with petroleum substances [5,21].

The MI is the main value derived from the modified Ames test. The MI is the initial linear slope of the dose-response curve, expressed as µl of a DMSO extract of the test substance per cultured plate of bacteria (dose) and the number of revertant colonies on the respective plates (response). Under the standardized conditions of the test, the MI correlated well with tumorigenicity of distillate petroleum substances in skin-painting tests in mice [5,6,21]. Subsequently the modified Ames test was standardized (ASTM E 1687) with a domain of applicability related mainly to distillate refinery streams and lubricating basestocks. A value of “1” for the MI is commonly used as a cut-off for distillate streams based on the observed relation of MI to tumours in skin-painting assays (ASTM E 1687).

Regarding RAE, Blackburn, et al. [6] reported that the MI for 8 samples of RAE in the modified Ames test ranged from 0.2 to 3.4, indicating a range of biological activity. These samples appeared to be similar to other petroleum substances in that the results of the modified Ames tests were related to the presence and level of DMSO-extractable PACs, which can vary with the crude oil and refining conditions. Therefore, as a prudent approach, individual samples could be assumed to be positive for carcinogenicity unless data (such as a low MI) indicate otherwise.

Given the observed variation of MI among samples of RAE, an evaluation of the association between MI and tumorigenicity was needed. It became apparent that this relation was different for RAEs compared to the established relationship for distillate steams and a cut-off of “1” for MI was not considered applicable for RAEs. As explained in subsequent sections, a lower MI has been selected as a more appropriate predictive cut-off for use with RAEs.

Other short-term assays of mutagenicity have been considered for petroleum hydrocarbons, but have not proved as useful as the modified Ames test. For

example, *in vivo* micronucleus evaluations were performed on bone marrow harvested at termination of the four subchronic dermal assays of RAEs [2]. No treatment-related increases in micronuclei were observed. Most micronucleus tests with other petroleum streams that contain higher amounts of PACs have also been negative, leading to the conclusion that PAC-containing petroleum substances generally have not produced chromosomal effects when tested in assays under *in vivo* conditions [22,33]. Overall these data indicate that RAEs did not cause chromosomal aberrations in an *in vivo* genotoxicity assay.

It is worth considering that the modified Ames assay was designed to screen samples with MIs ranging from zero up to several hundred; small variations among multiple tests on samples with MI less than one are not surprising given what one might expect due to biological variability. A round robin study, discussed below, was conducted to determine the extent of such variation and help determine if the variation would limit the use of the assay on samples with MIs less than one.

3. ASSESSMENT OF RAE HAZARD

3.1. RELATION BETWEEN THE MODIFIED AMES TEST AND DERMAL CARCINOGENICITY FOR RAE

Evaluation of the results from skin-painting studies on 11 RAE samples that had accompanying values for MI shows variable levels of biological activity. Using the generally accepted threshold (> 4%) for background tumour incidence, 5 samples were positive, 5 were negative, and 1 was equivocal. Details are provided in **Appendix 4**.

As stated previously, a MI of 1.0 from the modified Ames test is not considered an appropriate cut-off for estimating the potential carcinogenicity of RAEs. **Appendix 4** contains a summary of available data on RAEs that were tested in both a modified Ames assay and a skin-painting study in mice. Based on these data, a MI of 0.4 was chosen for use as a predictive cut-off for RAEs. And so, RAEs with MI <0.4 are considered non-carcinogenic and RAEs with MI >0.4 are considered to have potential carcinogenic activity.

3.2. MODIFIED AMES TEST – RAE ROUND ROBIN

As part of the process of establishing a value for the MI that could be used as a predictive cut-off for RAEs, it was first necessary to determine if the modified Ames test could be used for these materials in terms of repeatability. Therefore a round robin testing program was conducted in three laboratories. Three laboratories were selected to perform modified Ames tests on the same 7 RAE samples [12].

Three RAE producers sent samples of RAEs to a single member company dispensary. Each sample was divided into aliquots in identical containers and sent in duplicate (blinded, and uniquely coded) to each laboratory for conduct of the modified Ames test. One sample was sent twice to each laboratory (i.e. two identical sets of duplicate samples). Only CONCAWE H/TSG was aware of the identity of the samples. The samples were tested by each laboratory according to the lab's SOP for the modified Ames test.

Results were evaluated by H/TSG, and it was confirmed that repeatability was acceptable such that the modified Ames test could be used with RAE. Additional details of the round robin analyses are in **Appendix 5**.

4. CLASSIFICATION IMPLICATIONS

4.1. PREDICTIVE SCREENING FOR CARCINOGENICITY WITH MODIFIED AMES TEST

In the REACH registration dossiers, the modified Ames test was identified as a predictive tool for screening individual samples of RAEs for potential carcinogenicity. As described in the SOP in **Appendix 6**, the key difference in the application of the modified Ames test to RAEs compared to its use with OLBO is the temperature of the initial extraction of the sample with DMSO (45°C instead of 37°C). The other major difference is the use of a MI cut-off of 0.4 rather than 1.0.

4.2. CLASSIFICATION AGAINST EU DSD AND CLP CRITERIA

Five of the studies summarised in **Appendix 4** show a clear increase in the number of animals with dermal tumours and the majority of these studies also showed an increased incidence of malignant tumours. These data demonstrate clearly the ability of some RAEs to increase the incidence of both benign and malignant skin tumours in treated animals. It is recognised, however, that several samples did not show any carcinogenic activity when tested.

Based on the data presented in this report, it is considered that, as a 'worst case' default position, the criteria for classification as Category 3 (DSD) and Category 2 (CLP) would be met, reflecting the uncertainty around the cancer hazard potential of this group of aromatic extracts. Evaluation of the potential carcinogenic hazard of RAEs may be possible in the future via use of further hazard data and/or if an additional scientifically justified method can be identified to discriminate between carcinogenic and non-carcinogenic samples.

Based on a weight of evidence approach, it is recommended that all RAEs be considered as potentially carcinogenic and classified as noted above unless it can be shown that the substance has MI < 0.4. This OIN applies only to RAEs (CAS-numbers 64742-10-5 and 91995-70-9). The rationale for the selection of 0.4 as a cut-off of MI is presented in Section 3.1 and **Appendix 4** of this report.

As submitted in the REACH Dossier for the RAE category in 2010, the following OINs based on DSD regulation (OIN 9) and the Note based on CLP regulation (OIN 10) have been implemented.

OIN 9	The EC DSD classification as a carcinogen need not apply if it can be shown that the substance has MI less than 0.4 as measured by the test method described in ASTM E 1687-04 or if another predictive test demonstrates the substance is not carcinogenic. This OIN applies only to RAEs (CAS-numbers 64742-10-5 and 91995-70-9).
OIN 10	The EC CLP classification as a carcinogen need not apply if it can be shown that the substance has MI less than 0.4 as measured by the test method described in ASTM E 1687-04 or if another predictive test demonstrates the substance is not carcinogenic. This OIN applies only to RAEs (CAS-numbers 64742-10-5 and 91995-70-9).

5. CONCLUSIONS

Based on the reliability of the modified Ames test and the results from skin-painting studies, these data show that the modified Ames test can be used as a screening tool for assessing the potential carcinogenicity activity of RAEs. A cut-off value was determined to distinguish between carcinogenic and non-carcinogenic RAE samples. Data demonstrate that RAEs with an MI $\geq$ 0.4 demonstrated potential carcinogenic activity to mouse skin following chronic exposure. RAEs with a MI < 0.4 did not demonstrate a carcinogenic potential.

The results from the round robin study with RAEs support the utility of the modified Ames test as a predictive screening tool for potential carcinogenic activity of RAEs.

CONCAWE has prepared two classification notes: one OIN based on the DSD regulation (OIN 9) and one based on the CLP regulation (OIN 10).

6. GLOSSARY

ASTM	American Society of Testing and Materials
CAS	Chemical Abstract Service
CLP	Classification, Labelling and Packaging of chemicals
DAO	Deasphalted Oil
DMSO	Dimethyl sulfoxide
DSD	EU Dangerous Substances Directive
EINECS	European Inventory of Existing Commercial Chemical Substances
EU	European Union
H/TSG	Health Toxicology Subgroup
IARC	International Agency for Research on Cancer
MI	Mutagenicity Index
OIN	Oil Industry Note
OLBO	Other lubricant base oils
PACs	Polycyclic Aromatic Compounds
RAEs	Residual Aromatic Extracts
REACH	Registration, Evaluation, Authorisation of Chemicals
RN	Registry Number
SD	Standard deviation
SOP	Standard Operating Procedure
TBA	Tumour-Bearing Animals

7. REFERENCES

1. Ames, B.N. et al (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research 31, 347-364*

2. API (2011) Robust summary of information on aromatic extracts. (In preparation for submission to US EPA). Washington DC: American Petroleum Institute

3. ASTM (2010) Standard test method for determining carcinogenic potential of virgin base oils in metalworking fluids. ASTM E 1687. Philadelphia PA: American Society for Testing and Materials
http://www.astm.org

4. Bingham, E. et al (1980) Carcinogenic potential of petroleum hydrocarbons: a critical review of the literature. *J Environ PatholToxicol 3, 1-2, 483-563*

5. Blackburn, G.R. et al (1986) Predicting carcinogenicity of petroleum distillation fractions using a modified *Salmonella* mutagenicity assay. *Cell Biology and Toxicology 2, 1, 63-84*

6. Blackburn, G.R. et al (1996) Comparison of biological and chemical predictors of dermal carcinogenicity of petroleum oils. *Polycyclic Aromatic Compounds 11, 201-210*

7. Briggs, T. and Mackerer, C.R. (1996) The Chemistry and Toxicology of Petroleum Hydrocarbons. Washington D.C: AIHCE Professional Development Course

8. Chasey, K.L. and McKee, R.H. (1993) Evaluation of the dermal carcinogenicity of lubricant base oils by the mouse skin painting bioassay and other proposed methods. *J Appl Toxicol 13, 1, 57-65*

9. CONCAWE (1994) The use of the dimethyl sulphoxide (DMSO) extract by the IP 346 method as an indicator of the carcinogenicity of lubricant base oils and distillate aromatic extracts. Report No. 94/51. Brussels: CONCAWE

10. CONCAWE (2005) Classification and labelling of petroleum substances according to the EU dangerous substances directive (CONCAWE recommendations – July 2005). Report No. 6/05. Brussels: CONCAWE

11. CONCAWE (2010) Modified Ames test. Test substance: 16 coded samples. PLI study No. 109104. Study Conducted by Petrolabs, Inc. Brussels: CONCAWE

12. Cruzan, G. et al (1986) Systemic toxicity from subchronic dermal exposure, chemical characterization, and dermal penetration of catalytically cracked clarified slurry oil. *Toxicol & Ind Health, 2, 429-444*

13. Doak, S.M.A. et al (1985) Carcinogenic potential of hydrotreated petroleum aromatic extracts. *Brit J Ind Med, 42, 380-388*

14. EBSI (1997a) Microbial mutagenesis in *Salmonella* mammalian microsome preincubation assay using DMSO extraction modification. Test substance: brightstock extract (MRD-96-601). Study conducted for Exxon Company

International. Report No. 160125. East Millstone NJ: Exxon Biomedical Sciences Inc.

15. EBSI (1997b) Microbial mutagenesis in *Salmonella* mammalian microsome preincubation assay using DMSO extraction modification. Test substance: brightstock extract (MRD-96-657). Study conducted for Esso Italiana S.A. Report No. 165725. East Millstone NJ: Exxon Biomedical Sciences Inc.

16. EMBSI (2005) Two year dermal carcinogenicity study in mice - test substances MRD-96-596, MRD-96-597, MRD-96-598, MRD-96-599, MRD-96-601, MRD-96-657, MRD-96-661. EMBSI Environ. Toxicol. & Chem. Lab. Report No. 159611. Fairfax VA: ExxonMobil Lubricants & Petroleum Specialties

17. Gradiski, D. et al (1983) The carcinogenic effect of a series of petroleum-derived oils on the skin of mice. *Environmental Research 32, 258-268*

18. Kane, M.L. et al (1984) Toxicological characteristics of refinery streams used to manufacture lubricating oils. *Am J Ind Med 5, 3, 183-200*

19. King, D.J. (1991) 1156, 1157 and 1158: 2-year skin painting study. Toxicology Report No. 25-90-0275. Guildford: BP Group Occupational Health Centre

20. Mackerer, C.R. et al (2003) Petroleum mineral oil refining and evaluation of cancer hazard. *Appl Occup Environ Hyg 18, 11, 890-901*
PMID:14555442

21. McKee, R.H. et al (2010) Assessment of the *in vivo* cytogenetic potential of petroleum-derived materials. Presented at Society of Toxicology, March 7-11. Abstract number 1122 in *The Toxicologist*

22. Mobil (1987a) A modified Ames pre-incubation mutagenesis assay for determination of specific mutagenicity of the DMSO extract of Coryton bright stock extract. Mobil Environ. and Health Sci. Lab. Study No. 61755. Princeton NJ: Mobil Oil Corporation

23. Mobil (1987b) A modified Ames pre-incubation mutagenesis assay for determination of specific mutagenicity of the DMSO extract of bright stock extract CT10. Mobil Environ. and Health Sci. Lab. Study No. 61809. Princeton NJ: Mobil Oil Corporation

24. Mobil (1987c) A modified Ames pre-incubation mutagenesis assay for determination of specific mutagenicity of the DMSO extract of bright stock extract CT 11. Mobil Environ. and Health Sci. Lab. Study No. 61829. Princeton NJ: Mobil Oil Corporation

25. Mobil (1987d) A modified Ames pre-incubation mutagenesis assay for determination of specific mutagenicity of the DMSO extract of Mobilsol 40 (C.T. 28). Mobil Environ. and Health Sci. Lab. Study No. 62242. Princeton NJ: Mobil Oil Corporation

26. Mobil (1992) Dermal carcinogenicity study of four neat bright stock extracts, two bright stock extracts diluted with mineral oil, positive and negative control. Study conducted by Kettering Laboratory, University of Cincinnati. Study No. 62274, 62273, 62216, 62215, 62157, 62246, 62271 and 62272. Princeton NJ: Mobil Oil Corporation

27. Mobil (1998a) Modified Ames testing of ten oils from Mobil Corporation. PLI Study No. 98026. Study conducted by PetroLabs Inc. Report No. 67634ZB, 67634ZE, and 67634ZF. Princeton NJ: Mobil Oil Corporation

28. Mobil (1998b) Modified Ames testing of one oil sample. PLI Study No. 98043. Study conducted by PetroLabs Inc. Report No. 67647. Princeton NJ: Mobil Oil Corporation

29. Mobil (1998c) Modified Ames testing of one oil sample from Mobil Corporation. Study conducted by PetroLabs Inc. Report No. 67673. Princeton NJ: Mobil Oil Corporation

30. Mobil (2001) Dermal carcinogenicity study of 12 samples. (Only relevant RAEs are shown here.) Study conducted by Kettering Laboratory, University of Cincinnati. Study No. 67746. Fairfax VA: Mobil Business Resources Corporation

31. PetroLabs (2010) Method IP 346. http://www.petrolabs.com/IP346.htm, accessed 5-18-10. Ivyland PA: PetroLabs Inc

32. Przygoda, R.T. et al (1999) Assessment of the utility of the micronucleus test for petroleum-derived materials. *Mutation Res 438, 2, 145-153.*

33. Reddy, M.V. et al (1997) Correlation of mutagenic potencies of various petroleum oils and oil coal tar mixtures with DNA adduct levels *in vitro*. *Mutation Res 378, 89-95*

34. Roy, T.A. et al (1988) Correlation of mutagenic and dermal carcinogenic activities of mineral oils with polycyclic aromatic compound content. *Fundamental and Applied Toxicology 10, 3, 466-476*

35. Roy, T.A. et al (1996) Evaluation of physicochemical factors affecting dermal penetration and carcinogenic potency of mineral oils containing polycyclic aromatic compounds. *Polycyclic Aromatic Compounds 10, 1-4, 333-342*

APPENDIX 1 CAS RN AND EINECS DESCRIPTIONS FOR RESIDUAL AROMATIC EXTRACTS.

Residual aromatic extract Entries in the European Inventory of Existing Commercial chemical Substances (EINECS). These entries were also registered under the European Existing Substances Regulation [COUNCIL REGULATION (EEC) No 793/93].

EINECS number	CAS Registry Number
265-110-5	64742-10-5
Extracts (petroleum), residual oil solvent A complex combination of hydrocarbons obtained as the extract from a solvent extraction process. It consists predominantly of aromatic hydrocarbons having carbon numbers predominantly higher than C_{25}	
295-332-8	91995-70-9
Residues (petroleum), vacuum A complex combination of hydrocarbons obtained by solvent extraction of a vacuum-deasphalted residue. It consists predominantly of aromatic hydrocarbons having carbon numbers predominantly greater than C_{30}. This stream contains more than 5 wt. % of 4- to 6-membered condensed ring aromatic hydrocarbons	

APPENDIX 2 CLASSIFICATION CRITERIA FOR CARCINOGENICITY

Dangerous Substances Directive

1. Carcinogenic substances

For the purpose of classification and labelling, and having regard to the current state of knowledge, such substances are divided into three categories:

Category 1
Substances known to be carcinogenic to man. There is sufficient evidence to establish a causal association between human exposure to a substance and the development of cancer.

Category 2
Substances which should be regarded as if they are carcinogenic to man. There is sufficient evidence to provide a strong presumption that human exposure to a substance may result in the development of cancer, generally on the basis of:

- appropriate long-term animal studies,
- other relevant information.

Category 3
Substances which cause concern for man owing to possible carcinogenic effects but in respect of which the available information is not adequate for making a satisfactory assessment. There is some evidence from appropriate animal studies, but this is insufficient to place the substance in category 2.

1.1 The following symbols and specific risk phrases apply:

Categories 1 and 2:
Substances classified carcinogenic category 1 or 2 shall be assigned the symbol .T. and the risk phrase R45 May cause cancer. However, substances and preparations which present a carcinogenic risk only when inhaled, for example, as dust, vapour or fumes, (other routes of exposure e.g. by swallowing or in contact with skin do not present any carcinogenic risk), shall be assigned the symbol .T. and the risk phrase R49 May cause cancer by inhalation

Categories 3:
Substances classified as carcinogenic category 3 shall be assigned the symbol .Xn. and the risk phrase R40 Limited evidence of a carcinogenic effect

1.2. Comments regarding the categorisation of carcinogenic substances

The placing of a substance into category 1 is done on the basis of epidemiological data; placing into categories 2 and 3 is based primarily on animal experiments. For classification as a category 2 carcinogen either positive results in two animal species should be available or clear positive evidence in one species, together with supporting evidence such as genotoxicity data, metabolic or biochemical studies, induction of benign tumours, structural relationship with other known carcinogens, or data from epidemiological studies suggesting an association.

Category 3 actually comprises 2 subcategories:
(a) substances which are well investigated but for which the evidence of a tumour-inducing effect is insufficient for classification in category 2. Additional experiments would not be expected to yield further relevant information with respect to classification;
(b) substances which are insufficiently investigated. The available data are inadequate, but they raise concern for man. This classification is provisional; further experiments are necessary before a final decision can be made.

For a distinction between categories 2 and 3 the arguments listed below are relevant which reduce the significance of experimental tumour induction in view of possible human exposure.

These arguments, especially in combination, would lead in most cases to classification in category 3, even though tumours have been induced in animals:

- carcinogenic effects only at very high dose levels exceeding the maximal tolerated dose. The maximal tolerated dose is characterised by toxic effects which, although not yet reducing lifespan, go along with physical changes such as about 10 % retardation in weight gain,
- appearance of tumours, especially at high dose levels, only in particular organs of certain species known to be susceptible to a high spontaneous tumour formation,
- appearance of tumours, only at the site of application, in very sensitive test systems (e.g. i.p. or s.c. application of certain locally active compounds), if the particular target is not relevant to man,
- lack of genotoxicity in short-term tests in vivo and in vitro,
- existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g., hormonal effects on target organs or on mechanisms of physiological regulation, chronic stimulation of cell proliferation),
- existence of a species-specific mechanism of tumour formation (e.g. by specific metabolic pathways) irrelevant for man.

For a distinction between category 3 and no classification arguments are relevant which exclude a concern for man:

- a substance should not be classified in any of the categories if the mechanism of experimental tumour formation is clearly identified, with good evidence that this process cannot be extrapolated to man,
- if the only available tumour data are liver tumours in certain sensitive strains of mice, without any other supplementary evidence, the substance may not be classified in any of the categories,
- particular attention should be paid to cases where the only available tumour data are the occurrence of neoplasms at sites and in strains where they are well known to occur spontaneously with a high incidence.

APPENDIX 3 CLASSIFICATION, PACKAGING AND LABELLING REGULATION

1. Carcinogenicity

1.1 Definition

1.2.1. Carcinogen means a substance or a mixture of substances which induce cancer or increase its incidence. Substances which have induced benign and malignant tumours in well performed experimental studies on animals are considered also to be presumed or suspected human carcinogens unless there is strong evidence that the mechanism of tumour formation is not relevant for humans.

1.2 Classification criteria for substances

1.2.1. For the purpose of classification for carcinogenicity, substances are allocated to one of two categories based on strength of evidence and additional considerations (weight of evidence). In certain instances, route-specific classification may be warranted, if it can be conclusively proved that no other route of exposure exhibits the hazard.

Table 3.6.1

Hazard categories for carcinogens

Categories	Criteria
CATEGORY 1:	Known or presumed human carcinogens A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:
Category 1A:	Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or
Category 1B:	Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from: — human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or — animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals.
CATEGORY 2:	Suspected human carcinogens The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

(1) Note: See 3.6.2.2.4.

1.2.2. *Specific considerations for classification of substances as carcinogens*

1.2.2.1. Classification as a carcinogen is made on the basis of evidence from reliable and acceptable studies and is intended to be used for substances which have an intrinsic property to

cause cancer. The evaluations shall be based on all existing data, peer-reviewed published studies and additional acceptable data.
1.2.2.2. Classification of a substance as a carcinogen is a process that involves two interrelated determinations: evaluations of strength of evidence and consideration of all other relevant information to place substances with human cancer potential into hazard categories.

1.2.2.3 Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance.
Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated. Limited evidence in animals is provided when data suggest a carcinogenic effect, but are less than sufficient. The terms 'sufficient' and 'limited' have been used here as they have been defined by the International Agency for Research on Cancer (IARC) and read as follows:

(a) Carcinogenicity in humans
The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

- sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence;
- limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

(b) Carcinogenicity in experimental animals
Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals. The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

- sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an **appropriate combination of benign and malignant neoplasms** in (a) two or more species of animals or **(b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols**. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;
- limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

APPENDIX 4 RELATION OF MI TO INCIDENCE OF TUMOURS IN MOUSE SKIN PAINTING STUDIES WITH RESIDUAL AROMATIC EXTRACTS

Table 4-1 contains available data on samples of RAE that were tested in both the modified Ames assay and skin-painting assays in mice. The samples are arranged by increasing MI from the modified Ames test. The percent tumour-bearing animals (% TBA) in the skin-painting assay is used as an indication of whether a given RAE could be considered to be carcinogenic. The industry position has been to assume that two or more animals out of 50 tested (equal or greater than 4%) producing tumours is above the expected incidence of spontaneous tumours and needs to be considered as a positive result unless there are reasons to suspect that an observed tumour might be spontaneous.

By these criteria five samples with MI equal to or greater than 0.4 were considered carcinogenic. Of the remaining data points, 5 were negative and one is reported as being equivocal. From the study report, one of the TBA with this sample developed a squamous cell carcinoma that was seen in-life. It was first observed as a papilloma at ~Day 357 and progressed to a carcinoma at ~Day 532. The animal was euthanized on Day 538 and the carcinoma was confirmed histologically. No tumour was seen in-life on the second TBA. The second TBA was euthanized on Day 605 and a papilloma was confirmed during the histopathological examination. One TBA in a group of 40-50 mice is considered to represent the spontaneous rate of squamous cell tumours in mice. It has been suggested by Blackburn et al (1986) [5] that the true spontaneous rate is 1 to 2 TBA in a group of 50. In addition the tumour occurred very late in the study which is another factor suggesting that it was spontaneous in origin and likely not treatment related. Therefore the result with MRD-96-657 were considered to be equivocal and its carcinogenic potential could not be definitively determined [17].

Therefore, when the data in **Table 4-1** are evaluated, the point at which a consistent positive tumour response occurs is when the MI reaches 0.4. These data indicate that a MI value of 0.4 can serve as a reliable cut-point such that RAEs with MIs less than this value are likely not carcinogenic. Stated differently,

- RAEs with MI less than 0.4 are considered non-carcinogenic and
- RAEs with MI greater than or equal to 0.4 are considered to have potential carcinogenic activity.

Table 4-1 Comparison of MI in modified Ames test and percent of tumour-bearing animals (TBA) in skin-painting assays in mice for individual samples of RAE

Sample[a]	MI[b]	% TBA[c]	Study Duration (wk)	No. Mice per Group	Skin-Painting Study Reference	MI Study Reference
MRD-96-601	0	0	104	50	[17]	[15]
CRU 98039	0.1	0	78	50	[31]	[28]
CRU 98059	0.2	0	78	50	[31]	[29]
CRU 97084	0.1 to 0.3[d]	0	78	50	[31]	[28]
MRD-96-657	0.3	4[e]	104	50	[17]	[16]
CRU 97125	0.4	2	78	50	[31]	[28]
CRU 87049	0.4 to 0.6[f]	16	104	50	[27]	[25]
CRU 87476	0.7	20	104	50	[27]	[26]
CRU 98076	0.9	24	78	40	[31]	[30]
CRU 87040	1.1	12	104	[g]	[27]	[24]
CRU 86518	5.6	40	104	50	[27]	[23]

[a]) CRU numbers were the laboratory's internal tracking numbers for individual samples.
[b]) MI is mutagenicity index in the modified Ames test.
[c]) "% TBA" is the percent of tumour-bearing animals in skin-painting assays.
[d]) Two modified Ames assays were performed independently on CRU 97084, resulting in MIs of 0.1 and 0.3.
[e]) This incidence of tumours was considered equivocal. See text on the previous page for an explanation regarding spontaneous incidence of tumours.
[f]) MI in the available report for this sample was 0.4. The sample was subsequently retested in the same laboratory without formal study reports; the resulting MIs were as high as 0.6.
[g]) Number of mice per group was not available.

APPENDIX 5 SUMMARY OF ROUND ROBIN STUDY ON MODIFIED AMES TESTS WITH RAES

The mutagenic potential of a substance can be assessed with a reverse mutation test (Ames test). Since this test is not very well suited for highly lipophilic substances, including petroleum substances, a modified version was developed that aims to assess the mutagenicity of oil products (modified Ames test) by determining the MI. For virgin mineral hydrocarbon basestocks, this modified Ames test was calibrated against a large number of mouse skin painting studies to also provide an indication of carcinogenic potential. The numerical value of the MI can be used to differentiate between base oils that need to be classified as carcinogenic and those that need not. Although often used for the cancer classification of oil products other than virgin basestocks, the modified Ames test has not been validated for this purpose.

As described previously in this report, data on MI and skin-painting studies in mice are available for a number of RAEs, allowing definition of a value of MI that can be used to differentiate between carcinogenic and non-carcinogenic RAEs. However, since RAEs have different physico-chemical properties than base oils, questions arose regarding the routine use of the modified Ames test with RAEs. In particular, the test has not been validated with respect to repeatability with RAEs. Therefore a round robin study was conducted to investigate repeatability (intra- and inter-laboratory) of the modified Ames test with RAEs.

Samples of RAEs, covering both CAS numbers used for RAEs (i.e. 64742-10-5 and 91995-70-9), were provided by three manufacturers to a single member company dispensary. At least 500 g of each sample were requested. Multiple aliquots of each sample were put into identical containers, uniquely coded, and sent blind to three participating laboratories that conducted the modified Ames' test. Each laboratory received two aliquots of each sample. An additional two aliquots of one sample were sent to each laboratory (i.e. two identical sets of duplicate samples). Thus each laboratory received 16 aliquots, of which 4 were taken from one sample and the remaining 12 were duplicate aliquots from the remaining 6 samples. CONCAWE H/TSG had the codes to identify the samples and aliquots; the laboratories did not.

Each laboratory performed the modified Ames test on the aliquots as received. The tests were to be performed in accordance with the lab's SOP that adhered to the process outlined in ASTM E1687-04. CONCAWE H/TSG requested the sample be handled and extracted at 45°C. The CONCAWE H/TSG evaluated the results of these tests for the intra-laboratory variation (mean and standard deviation based on multiple aliquots per sample). Inter-laboratory variation was evaluated using means and standard deviations of the mean MI of each sample at each laboratory. The calculated variations in MI were then compared to the results as presented in ASTM E1687 for virgin mineral hydrocarbon base oils, with the expectation that the variation in MI for the RAEs might be somewhat larger due to the smaller number of samples as well as the physicochemical properties of the RAEs.

The observed MIs for each aliquot are summarized in **Table 5-1** (intra-laboratory variation) and the variation among the three laboratories is summarized in **Table 5-2**. Note that calculations of means and standard deviations are shown to the nearest hundredth as a means of showing the variation among the values; final values are rounded to the nearest tenth, the more appropriate procedure for MIs. This format is used here because it matches the format used in ASTM E1687-04 for presentation of similar data.

Table 5-1 Values for MI on 7 samples of RAEs as determined at 3 laboratories on multiple aliquots of each sample. Means ± standard deviation (SD) are also shown.[1]

	Laboratory		
Sample	A	B	C
1	0.1, 0.1, 0.1, 0.0 0.07 ± 0.06	0.3, 0.3, 0.3, 0.4 0.32 ± 0.03	0.3, 0.3, 0.1, 0.1 0.21 ± 0.13
2	0.1, 0.1 0.12 ± 0.02	0.4, 0.4 0.41 ± 0.03	0.1, 0.3 0.19 ± 0.15
3	0.1, 0.1 0.10 ± 0.01	0.2, 0.3 0.29 ± 0.07	0.3, 0.3 0.28 ± 0.02
4	0.2, 0.2 0.23 ± 0.01	0.4, 0.4 0.41 ± 0.03	0.3, 0.2 0.28 ± 0.06
5	0.2, 0.1 0.17 ± 0.06	0.5, 0.5 0.48 ± 0.05	0.3, 0.2 0.28 ± 0.10
6	0.0, 0.1 0.05 ± 0.09	0.2, 0.3 0.25 ± 0.06	0.3, 0.4 0.33 ± 0.02
7	0.0, 0.1 0.06 ± 0.07	0.3, 0.4 0.36 ± 0.07	0.3, 0.2 0.24 ± 0.12

1) Values for MI are rounded to nearest tenth, as is appropriate. Values for mean and SD were left at nearest hundredth to demonstrate the variation in MI more clearly.

Table 5-2 Comparison of mean values of MI for each sample as determined at three laboratories.

Sample	A	B	C	Mean ± SD
1	0.1	0.3	0.2	0.20 ± 0.13
2	0.1	0.4	0.2	0.24 ± 0.15
3	0.1	0.3	0.3	0.22 ± 0.11
4	0.2	0.4	0.3	0.31 ± 0.09
5	0.2	0.5	0.3	0.31 ± 0.16
6	0.1	0.2	0.3	0.21 ± 0.15
7	0.1	0.4	0.2	0.22 ± 0.15

1) Values for MI are rounded to nearest tenth, as is appropriate. Values for mean and SD were left at nearest hundredth to demonstrate the variation in MI more clearly.

It can be seen from these tables that the MIs from Laboratory A tended to be lower than those from the other laboratories and the MIs from Laboratory B tended to be the highest. The SOP specified 2 plates per dose group per extract. In contrast, Laboratory A performed the assays with only one set of plates per dose group per extract (1 plate per dose group per each aliquot

from CONCAWE H/TSG). For this and other reasons, the MIs from Laboratory A were considered questionable.

Both Laboratory B and Laboratory C exceeded the specifications in the SOP by performing triplicate assays on duplicate plates for each dose group for each (6 plates per dose group per each aliquot from CONCAWE H/TSG). However, due to difficulties with a supplier of key reagents, Laboratory B altered the extraction procedure used to derive the S-9 fraction used for metabolic activation in the modified Ames test. The MIs from this laboratory tended to be higher than expected compared to previous tests (personal communication, lab director), perhaps as a result of the altered procedure for S-9.

Therefore the accuracy of MIs from Laboratories A and B was questionable due to identifiable reasons. Although the accuracy of the values for these MIs was in doubt, the variation among these values within each laboratory was considered to be at least representative. Variation among the laboratories might have been greater than it would have been if all three laboratories had used identical procedures.

Given these points, it can be concluded from **Table 5-1** that values for MI from multiple analyses of the same sample at the same laboratory were generally within 0.1 revertants/µL/plate. Inter-laboratory variation was apparent in **Table 5-2**, but the standard deviation of MIs for each sample analysed at this limited number of laboratories was ~0.1. These results supported the use of the modified Ames test with RAEs.

For comparison, ASTM E1687-04 contains a summary of modified Ames tests performed on multiple samples of virgin base oils at six different laboratories. Two of those samples had MI less than 1.0. The reported MIs for the first of these two samples ranged from 0.1 to 0.7 with a mean and standard deviation of 0.3 ± 0.2. The range of MIs for the second sample was 0.5 to 1.0; the mean and standard deviation were 0.8 ± 0.2. The variation observed with the smaller number of analyses of RAEs (3 laboratories versus 6) was no greater than the variation observed with virgin base oils.

Three suggestions are presented here for the conduct of modified Ames tests with RAEs.

1) The inter-laboratory differences summarized above point to the need to establish definitive procedures at a given laboratory before modified Ames tests are performed at that laboratory on RAEs.
2) The use of one RAE as a reference sample to be used with any assay on a RAE is also suggested. This approach would be similar to the use of reference base oil in modified Ames tests on samples of base oils.
3) Multiple MIs determined on a given sample can vary by 0.1. Therefore, assigning precision greater than 0.1 to a MI (such as a value to the nearest hundredth) is not recommended.

APPENDIX 6: SUGGESTED MODIFIED AMES STANDARD OPERATING PROCEDURE

Standard Operating Procedure

Study Title: **Modified Ames Test in *Salmonella* using DMSO Extraction Modification**

Applicable for substances: Residual Aromatic Extract (CASRN 64742-10-5 and 91995-70-9)

Contents

1. INTRODUCTION

1.1. PURPOSE

This study will be conducted in order to evaluate the MI of the test substances in one selected strain of *Salmonella typhimurium* for residual aromatic extract (CASRN 64742-10-5 and 91995-70-9).

1.2. SPONSOR

This study will be conducted for:

1.3. TESTING FACILITY

The study will be conducted by:

1.4. COMPLIANCE

This study will be conducted in general agreement with the following standard:

American Society of Testing and Materials (ASTM E 1687-04), The Standard Test Method for Determining Carcinogenic Potential of Virgin Base Oils in Metal working Fluids.

1.5. JUSTIFICATION FOR SELECTION OF TEST SYSTEM

TA98, a special strain of *Salmonella*, has historically been used for presumptive identification of genotoxic mutagens (Ames et al., 1975, Maron and Ames, 1983) and is the strain of choice for microbial gene mutation studies when testing petroleum-derived products (Blackburn et al., 1986 and 1996).

1.6. JUSTIFICATION OF DOSING ROUTE

Exposure by the pre-incubated route was demonstrated previously to produce more reliable results when testing certain petroleum-derived products (Blackburn et al., 1986).

2. TEST SUBSTANCES

2.1. SUBSTANCE IDENTIFICATION

Residual extract aromatic (State applicable CASRN 64742-10-5 or 91995-70-9).

2.2. VEHICLE

Dimethyl sulfoxide (DMSO), extraction agent used in the preparation of aromatic enriched oil fractions for mutagenicity testing.

2.3. CARRIER

Top Agar (agar overlay containing minimal biotin and histidine)

2.4. POSITIVE CONTROL SUBSTANCE

The positive control substance will be considered under the conditions of the assay to be stable for the duration of the assay in that it performed in a manner consistent with published results (ASTM, 1998). The positive control substance should be benzo(a)pyrene or other substance as vacuum distillate.

2.5. CHARACTERIZATION OF TEST SUBSTANCE

The stability, identity, solubility, strength, purity (other than that the substance was to be considered 100% pure for testing purposes) and composition or other characteristics which will appropriately identify the test substance will not be performed.

2.6. TEST SUBSTANCE/VEHICLE MIX RETENTION SAMPLES

No retention samples will be taken.

3. TEST SYSTEM

3.1. TESTER STRAIN

The *Salmonella typhimurium* strain used in this assay is derived from an original stock could be supplied by B.N. Ames, University of California, Berkeley, and is stored as a frozen permanent. The strain is designated TA98.

3.2. CULTURE AGE AT INITIATION OF DOSING

TA98 is grown overnight (approximately 16 hr.) in a nutrient broth at $37\pm2^{o}C$ in a shaking incubator (100 rpm). Then, it is diluted 1/5 and allowed to grow for three more hours. The culture was prepared from frozen permanent stock, stored at or below $-75^{o}C$, labelled by strain number.

3.3. METABOLIC ACTIVATION (S9)

S9, from the livers of Aroclor 1254 pre-treated Golden Syrian hamsters, will be used. Preparation of the S9 mix for the assay will follow the method described in the

ASTM E1687-10. This mixture could be done by the lab or bought at specific supplier.

4. EXPERIMENTAL DESIGN

4.1. PREPARATION OF TESTER STRAINS

On the day before the assay, a frozen culture will be used to inoculate a nutrient broth culture tube. This culture will be incubated for 8-16 hours at 37 ± 2°C. Following the initial incubation period, 2 mL of the culture will be inoculated into 8 mL of nutrient broth and incubated for an additional three hours. The 3 hour culture will then be kept on ice during the dosing procedure.

4.2. HANDLING AND PREPARATION OF THE TEST SUBSTANCES/ POSITIVE CONTROL SUBSTANCE

4.2.1. Handling and Preparation of the Positive Control Substance

The positive control will be heated to 45°C (to decrease the viscosity of the material, if required) and will be mixed thoroughly so as to achieve a homogeneous mixture prior to dispensing.

On the day prior to/or on the day of dosing, 2.5 mL of DMSO will be added to three separate 0.5 mL samples each of positive control. The mixtures will be vortexed and placed in a 45°C waterbath. They will be removed and vortexed every five minutes for a total of thirty minutes to ensure thorough contact between the oil and DMSO layers.

The fractions will be separated by centrifugation using a clinical centrifuge at 1000 rpm for 10 minutes. The lower DMSO layers are harvested. The DMSO layer obtained from the positive control extraction will be diluted 1:1 with DMSO.

4.2.2. Handling and Preparation of the Test Substance(s)

The RAE will be heated to 45°C to decrease the viscosity of the material and will be mixed thoroughly so as to achieve a homogeneous mixture prior to dispensing.

On the day prior to/or the day of dosing, 2.5 mL of DMSO will be added to three separate 0.5 mL samples each of test substance (RAE). The mixtures will be vortexed, placed in a waterbath and heated to approximately 45°C, decreasing the viscosity of the material, which will allow for thorough mixing of the substance with DMSO. They will be removed and vortexed every five minutes for a total of thirty minutes to ensure thorough contact between the oil and DMSO layers.

The fractions will be separated by centrifugation for instance at 200g for 10 minutes. The lower DMSO layers are harvested.

Vehicle dose will be kept below levels, which are toxic for the tester organism.

Note: Volumes of DMSO and test substance/positive control substance may be adjusted based on substance availability.

4.3. EXPERIMENTAL GROUPS

There will be one treatment set for the test substance and positive control substance. The set will have exogenous metabolic activation (8x S9) added. It will include 5 dose groups/extract for the test substance and 8 dose groups for the positive control, plus a vehicle control. There will be 2 plates per dose group per extract. Test doses for this assay will be 12, 24, 36, 48, and 60 μL/plate.

Example of preparation
1) 60 µl dose
dose 60 µl x 2 tubes = 120 µl used 400 µl -120 µl = 280 µl of extract left
- add 70 µl DMSO to remaining 280 µl of extract in tube & mix well
- results in 350 µl of diluted extract equal to the 48 µl dose.

2) 48 µl dose
dose 60 µl x 2 tubes = 120 µl used 350 µl -120 µl = 230 µl of extract left
- add 77 µl DMSO to remaining 230 µl of extract in tube & mix well
- results in 307 µl of diluted extract equal to the 36 µl dose.

3) 36 µl dose
dose 60 µl x 2 tubes = 120 µl used 307 µl -120 µl = 187 µl of extract left
- add 92 µl DMSO to remaining 187 µl of extract in tube & mix well
- results in 279 µl of diluted extract equal to the 24 µl dose.

4) 24 µl dose
dose 60 µl x 2 tubes = 120 µl used 279 µl -120 µl = 159 µl of extract left
- add 159 µl DMSO to remaining 159 µl of extract in tube & mix well
- results in 318 µl of diluted extract equal to the 12 µl dose.

5) 12 µl dose
dose 60 µl x 2 tubes = 120 µl used 318 µl -120 µl = 198 µl of extract left

The positive control doses can be considered as 3, 6, 9, 12, 15, 18, 21 and 24 μL/plate, as a starting point. Each test plate will be identified by the use of an imprinted label specifying tester strain, test substance, dose and presence of metabolic activation as well as the study number.

The controls for this study may serve as concurrent controls for another study.

If a positive response is observed, testing at closer dose intervals may be conducted to further define the activity of the test substance. This would be done subsequent to consultation with the Sponsor Representative, and receipt of authorization.

4.4. ADMINISTRATION OF TEST SUBSTANCE/POSITIVE CONTROL SUBSTANCE

A 60 µl sample of the extract is dispensed into sterile glass tubes; after 30 minutes the S9 mixture is added and subsequently the bacteria. The mixture will be transferred to an Orbit shaker at 150 rpm and incubated at 37±2°C for 20 to 30 minutes. The vehicle control will be handled in the same manner as the extract. Top agar will be cooled to approximately 40°C prior to adding the biotin and histidine

solution. It will then be added and the mixture vortexed and then poured into petri dishes containing a layer of minimal agar medium. After the agar has solidified, the plates will be incubated for 2 days at 37±2°C.

4.5. OBSERVATIONS AND TERMINATION

The plates may be counted immediately following incubation or may be refrigerated for up to 3 days prior to evaluation. If the vehicle mean revertant colony counts are between 30 and 60 revertants per plate, all plates will be evaluated for gross toxic effects and total revertant colony numbers. If the mean number of vehicle control revertant colonies is below 30, the plates will be incubated at room temperature overnight and read the following day. Colonies will be counted manually. MI values will be calculated according to methods described in the section “Data interpretation”.

4.6. VALIDATION OF MUTAGENICITY ASSAY

In order for an assay to be considered valid, certain criteria should be met.

1. The solvent control values should fall between 30 and 60 revertants per plate.
2. The reference oil should induce at least a doubling of revertants over the solvent control

4.7. DATA INTERPRETATION

In accordance with published procedures (Snedecor and Cochran, 1989), the mean revertant colony count and standard deviation for each dose point will be determined.

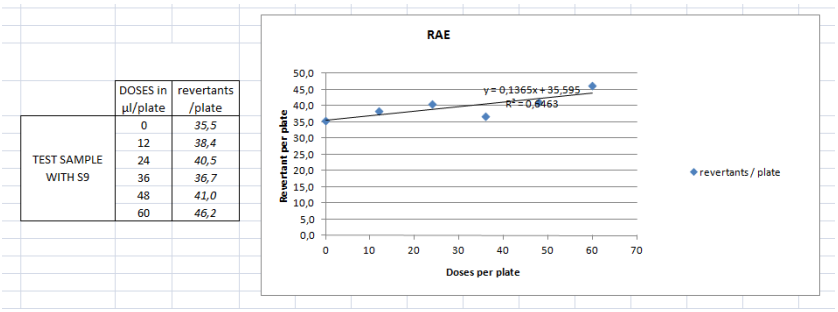
If the assay meets the criteria in section 4.6, a plot of colony counts or their means against dose is used to generate a dose response curve for mutagenesis. Linear regression analysis of this curve produces a slope (coefficient of the x-term of the regression equation) with units of revertants/µL DMSO extract. This slope is the fundamental measurement obtained through the use of this test method.

DMSO extracts of all oils should be diluted sufficiently that the dose-response for mutagenicity is linear over at least four doses

The slope of the initial linear region of the dose response curve will be used to generate a MI value for the test substances.

Excel spreadsheet as indicated in the **Figure 1** below could be easily done to estimate the slop

Figure 1 Example on linear regression analysis with excel spreadsheet



Toxicity is defined as a notable reduction in the background lawn and/or a greater than 50% reduction in the mean number of revertant colonies when compared to the vehicle control. When the mean number of revertant colonies for a test substance concentration is greater than or equal to two times the vehicle control, toxicity may also be defined as a greater than 50% reduction in the number of revertant colonies at concentrations higher than the concentration that induced the largest increase in revertant colonies. A statistically significant ($p<0.05$) reduction in mean number of revertant colonies may be used to define toxicity.

5. REPORTS

After termination of the study, an abbreviated final report, which includes the following information, will be submitted:

- Summary of the results.
- Deviations from experimental design, if any, and an estimate of the effects on the study objectives.
- Slope of the MI values for test substances based on section 4.7
- Analytical data on the substance

6. RECORDS

All appropriate substances, methods and experimental measurements required in this protocol will be recorded and documented in the raw data. Any changes, additions, or revisions of this protocol must be approved by the requestor. These changes will be documented in writing, including the date and the justification for the change.

The protocol, final report, computer generated listing(s) of raw data and supporting documentation will be maintained in Archives. The bacterial plates will not be saved, as extraordinary measures would be required.

7. REFERENCES

American Society of Testing and Materials (ASTM E-1687). (2010). The Standard Test Method for Determining Carcinogenic Potential of Virgin Base Oils in Metalworking Fluids.
http://www.astm.org

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Method for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test. Mutation Research. 31:347-364.

Blackburn, G.R., Deitch, R.A., Schreiner, C.A., and Mackerer, C.R. (1986). Predicting Carcinogenicity of Petroleum Distillation Fractions using a Modified Salmonella Mutagenicity Assay. Cell Biol. Toxicol. 2:63-84.

Blackburn, G.R., Roy, T.A., Bleicher Jr., W.T., Reddy, M.V., and Mackerer, C.R. (1996). Comparison of Biological and Chemical Predictors of Dermal Carcinogenicity of Petroleum Oils. Journal of Polycyclic Aromatic Compounds. 11:201-210.

Maron, D.M. and Ames, B.N., (1983). Revised Methods for *Salmonella*/Mutagenicity Test. Mutation Research, 113:173-215

Myers, L.E., Sexton, N.H., Southerland, L.I. and Wolf, T.J. (1981). Regression Analysis of Ames Test Data. Env. Mut. 3:575.

Snedecor and Cochran. Statistical Methods. 8th Edition, Iowa State University Press, Ames, Iowa, 1989.

SOP Annex 1: CAS and EINECS descriptions for RAEs

Residual aromatic extract Entries in the European Inventory of Existing Commercial chemical Substances (EINECS). These entries were registered under the European Existing Substances Regulation [COUNCIL REGULATION (EEC) No 793/93]

EINECS number	CAS Registry Number
265-110-5 **Extracts (petroleum), residual oil solvent** A complex combination of hydrocarbons obtained as the extract from a solvent extraction process. It consists predominantly of aromatic hydrocarbons having carbon numbers predominantly higher than C_{25}	64742-10-5
295-332-8 **Residues (petroleum), vacuum** A complex combination of hydrocarbons obtained by solvent extraction of a vacuum-deasphalted residue. It consists predominantly of aromatic hydrocarbons having carbon numbers predominantly greater than C_{30}. This stream contains more than 5 wt. % of 4- to 6-membered condensed ring aromatic hydrocarbons	91995-70-9

CONCAWE
Boulevard du Souverain 165
B-1160 Brussels
Belgium

Tel: +32-2-566 91 60
Fax: +32-2-566 91 81
e-mail: info@concawe.org
website: http://www.concawe.org

ISBN 978-2-87567-014-4