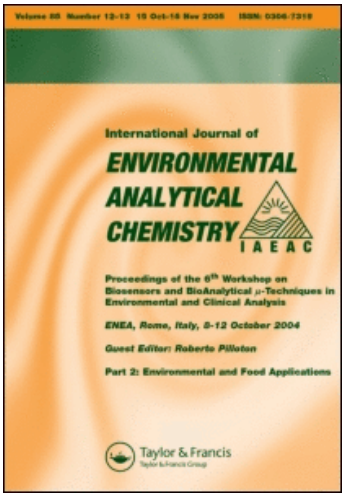


International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

# The Effect of Diesel Fuel Reformulation on the Exhaust Measured by Ames Mutagenicity and DNA AddUcts

Sanna Isotalo[a]; Terhi Kuljukka-Rabb[a]; Leena Rantanen[b]; Seppo Mikkonen[b]; Kirsti Savela[a]
[a] Finnish Institute of Occupational Health, Helsinki, Finland [b] Fortum Oil and Gas Oy, Finland

Online publication date: 17 September 2010

**To cite this Article** Isotalo, Sanna , Kuljukka-Rabb, Terhi , Rantanen, Leena , Mikkonen, Seppo and Savela, Kirsti(2002) 'The Effect of Diesel Fuel Reformulation on the Exhaust Measured by Ames Mutagenicity and DNA AddUcts', International Journal of Environmental Analytical Chemistry, 82: 2, 87 — 95
**To link to this Article: DOI:** 10.1080/03067310290024139
**URL:** http://dx.doi.org/10.1080/03067310290024139

*Intern. J. Environ. Anal. Chem.*, Vol. 82, No. 2, pp. 87–95

Taylor & Francis
Taylor & Francis Group

# THE EFFECT OF DIESEL FUEL REFORMULATION ON THE EXHAUST MEASURED BY AMES MUTAGENICITY AND DNA ADDUCTS

SANNA ISOTALO[a,*], TERHI KULJUKKA-RABB[a], LEENA RANTANEN[b], SEPPO MIKKONEN[b] and KIRSTI SAVELA[a]

[a]*Finnish Institute of Occupational Health, Topeliuksenkatu 217B, FIN-00250 Helsinki, Finland;* [b]*Fortum Oil and Gas Oy, Finland*

*(Received 10 October 2000; In final form 23 August 2001)*



In this study we compared two reformulated and one standard European Diesel fuel between particulate soluble organic fraction of PAH, mutagenic activity, and DNA adduct formation. Adducts were analyzed *in vitro* calf thymus DNA with and without the metabolic activation system and measured by $^{32}$P-postlabeling method. PAH-DNA adduct formation and mutagenicity by Ames test were compared. Both biological tests showed that reformulation decreased genotoxic PAH compounds in diesel particle emission.

*Keywords:* Diesel particle exhaust; Polycyclic aromatic hydrocarbons; DNA adducts; Diesel reformulation; $^{32}$P-postlabeling

## INTRODUCTION

Number of Diesel powered vehicles have increased and thus cause concern about the health effects of exhaust emission. According to IARC, exposure to diesel emission is probably carcinogenic to humans[1]. PAH and their substituted derivatives are an important class of organic compounds in the particulate phase. It has been shown that unsubstituted, parent PAH as well as nitrated, oxygenated and alkylated PAH contribute to the mutagenicity and carcinogenicity of diesel exhaust[2]. PAH bound to diesel particulate material are bioavailable to the cells and are metabolically activated to the reactive intermediates forming DNA adducts. DNA adducts are thought to be critical events in the initiation of chemically induced cancers, and adducts are important markers for studying of DNA damaging reactive chemicals inside the cell[3]. DNA adducts are the first sign of genotoxic effects in animal and human cells, and are thus valuable biomarkers of exposure to PAH and nitro-PAH in particulate.

In this study, we investigate two sets of Diesel particulate samples derived from two reformulated and one standard European diesel fuel. Fourteen PAH concentrations, DNA adduct forming potency and mutagenic activity were compared between

*Corresponding author. Fax: +358-9-47472114. E-mail: sanna.isotalo@occuphealth.fi

ISSN 0306-7319 print © 2002 Taylor & Francis Ltd
DOI: 10.1080/03067310290024139

particulate samples. Furthermore, differences in the activation of PAH and nitro-PAH derived from Diesel extracts in biological test systems were evaluated.

## EXPERIMENTAL

### Chemicals

Microccocal nuclease (from *Staphylococcus aureus*), nuclease P1, hypoxanthine, and xanthine oxidase were purchased from Sigma Chemical Co., St. Louis, MO, USA. Calf thymus DNA was from Merck, Darmstadt, Germany. Standard Reference Material (SRM, diesel particulate matter 1650) was supplied by the National Institute of Standards and Technology, Gaithersburg MD, USA. Benzo[a]pyrene and 1-nitropyrene were from Accu Standard New Haven, CT, USA. The S9 activation mixture was an in-house preparation from Aroclor 1254 induced male Wistar rats. Phosphodiesterase (from calf spleen) was from Boehringer Mannheim, GmbH, Germany. T4 polynucleotide kinase was from United States Biochemical, Cleveland, OH, USA. [$\gamma$-$^{32}$P]ATP 7000 Ci/mmol was purchased from ICN Pharmaceuticals, Inc., Irvine, CA, USA. All other chemicals and solvents were of analytical grade.



### Sample collection and determination of PAH

Exhaust particulate matter was collected from Diesel passenger car (Toyota, IDI engine) without oxidation catalyst using the modified European transient exhaust emission test procedure (ECE) and Extra Urban Driving Cycle (EUDC) after each other[4, 5]. Particulate emission from an European standard fuel (EN97; sulfur 430 mg/kg, total aromatics 36%, cetane number 49) and two reformulated fuels (RD1 and RD2; sulfur 27 and 1 mg/kg, total aromatics 19 and 2%, cetane number 56 and 54, respectively) was used during the test procedure. Sampling was performed identically twice (Set 1 and Set 2) for each Diesel fuel. Exhaust particles were collected through dilution tunnel on Teflon coated glass fiber filters. In order to get enough soluble organic fraction for the biological tests from the particulate mass, several identical test runs (from 4 to 24, depending on fuel tested) were repeated. Particulate samples from each testing day and fuel were collected and the sample extracts of each individual fuel were combined to give one sample per fuel. Combined particulate samples were extracted with Dichloromethane (DCM) using Soxhlet apparatus. The amount of soluble organic fraction (SOF) was calculated as filter weight difference. Dichloromethane of SOF extracts was changed, except for HPLC-analysis, to dimethylsulfoxide (DMSO) under nitrogen before using for the biological tests.

Concentrations of 14 PAH in SOF extracts were analyzed using HPLC equipped with time programmed wavelengths of fluorescence detector (Waters Corp. Milford, MA) according to NIOSH method[6]. The extracts were applied to a 5 µm, 4.6 mm × 250 mm Vydac RP18 column and eluted with water acetonitrile at flow rate 1 ml/min. EPA 610 PAH mixture containing 14 PAH compounds was used an external standard. 1-Nitropyrene (1-NP) was measured by GC/MS analysis from three extracts of Set 1 according to Nishioka *et al.*[7]. Briefly, analysis were carried out in triplicate with extracts (2 µg/ml) spiked with deuterated d9-1-nitropyrene. The analytical approach of 1-NP included normal phase silica HPLC-fractionation (7 µm, 10 mm × 250 mm column, flow rate 3.6 ml/min, with 254 nm) before GC/MS

analysis with negative chemical ionization (NCI). The identification and quantification (correct retention time, ions and ion abundance ratios) was based on the use of internal standard[7].

### Mutagenicity of Diesel extracts by Ames test

The SOF extracts were tested for mutagenicity in *Salmonella typhimurium* according to method described by Maron and Ames[8]. The Ames test was performed with the strains TA98 and YG1021 without external metabolic activation. TA98 strain is sensitive to mutagens causing frame-shift mutations, and YG1021 is a derivative of TA98 with an enhanced production of nitroreductase enzyme. 4-nitroquinoline-1-oxide was used as a positive control. SOF extracts of Set 1 were tested on triplicate plates with doses corresponding to 8, 16, 30, 63, 125 and 250 μg SOF per plate. SOF extracts of Set 2 were tested on quadruple plates in strain TA98 and YG1021 with doses of 0, 9.4, 19, 40, 75, 150 and 300 μg SOF, and 4.7, 9, 19, 38, 75 and 150 μg SOF per plate, respectively. Mutagenic activity was calculated of the positive slopes of dose response curves using the regression coefficient $B$ (when $y = A + Bx$). Mutagenicity was indicated as revertant colonies per mg SOF (Rev/mg SOF).



### Activation of PAH and nitro-PAH in CT DNA

The activation of PAH and nitro-PAH to DNA reactive intermediates was conducted by incubating the particulate extracts with CT DNA in oxidative and reductive reaction conditions. Benzo[a]pyrene, 1-nitropyrene, and Standard Reference Material 1650 were used as positive controls. *In vitro* activations were performed as described in Lewtas *et al.* 1981[9]. Briefly, oxidative activation of PAH was carried out in test tubes by incubating the S9 mixture and CT DNA at 37°C for 4 h. Reductive activation of nitro-PAH was carried out inside an anaerobic chamber under flow of nitrogen by incubating the hypoxanthine and xathine oxidase and CT DNA at 37°C for 4 h. After the incubations CT DNA was treated with RNAse and proteinase and extracted with phenol and chloroform.

### $^{32}$P-Postlabeling of DNA adducts

Four micrograms of CT DNA obtained from the activation reactions was taken to determine bulky aromatic DNA adducts by the $^{32}$P-postlabeling method[10]. DNA was digested to mononucleotides at 37°C for 3.5 h with micrococcal nuclease (0.2 U) and spleen phosphodiesterase (2 μg). The modified nucleotides of DNA digest were enriched with the butanol extraction and adducts were labeled by using T4 polynucleotide kinase (4.8 U) and 40 $\mu$Ci [$\gamma$-$^{32}$P] ATP. After two-dimensional chromatographic separation on the PEI–cellulose thin-layer plates, DNA adducts were visualized by autoradiography and quantified by Cerenkov counting.

## RESULTS

### PAH concentration in Diesel extracts

The concentrations of 14 PAH compounds in Diesel particulate extracts analyzed by HPLC from Set 1 and Set 2, and 1-nitropyrene by GC/MS from Set 1 are shown in

TABLE I Concentrations of PAH and 1-nitropyrene (ng/mg SOF) analyzed from two sets of Diesel particulate extracts RD2, RD1 and EN97

| *PAH compound* (ng/mg SOF) | *Set 1* | | | *Set 2* | | |
|---|---|---|---|---|---|---|
| | *RD2* | *RD1* | *EN97* | *RD2* | *RD1* | *EN97* |
| Fluorene [a] | 2.8 | 7.5 | 13.1 | 4.6 | 8.3 | 13.4 |
| Phenanthrene[a] | 492.4 | 662.9 | 731.1 | 551.8 | 607.9 | 796.6 |
| Anthracene[a] | 43.5 | 66.2 | 66.5 | 50.1 | 60.4 | 70.8 |
| Fluoranthene[a] | 763.4 | 932.7 | 751.4 | 580.2 | 653.7 | 583.0 |
| Pyrene[a] | 764.6 | 1553 | 931.8 | 573.9 | 1037 | 738.6 |
| Benzo[a]anthracene[b] | 54.6 | 73.7 | 97.1 | 40.2 | 55.4 | 71.3 |
| Chrysene[a] | 65.1 | 91.5 | 121.7 | 58.5 | 86.7 | 173.0 |
| Benzo[e]pyrene[a] | 93.5 | 150.2 | 119.3 | 87.7 | 109.8 | 99.7 |
| Benzo[a]pyrene[b] | 57.4 | 79.5 | 48.8 | 57.3 | 72.8 | 38.4 |
| Benzo[b]fluoranthene[c] | 62.5 | 87.7 | 73.9 | 50.3 | 59.1 | 43.9 |
| Benzo[k]fluoranthene[c] | 23.5 | 31.4 | 26.8 | 17.3 | 22.5 | 16.7 |
| Indeno [1,2,3-cd]pyrene[c] | 49.2 | 60.2 | 39.1 | 51.1 | 57.9 | 30.6 |
| Benzo[ghi]perylene[a] | 87.9 | 105.7 | 51.6 | 83.5 | 88.9 | 40.0 |
| Dibenz[a,h]anthracene[b] | 4.2 | 5.1 | 4.3 | 0 | 0 | 0 |
| 1-nitropyrene[c] | 26.0 | 27.3 | 34.0 | | | |
| Total | 2366 | 3657 | 2932 | 2206 | 2920 | 2716 |

[a]Non-carcinogenic to humans (class 3), [b]Probably carcinogenic to humans (class 2A), [c]Possibly carcinogenic to humans (class 2B) according to IARC Monograph [11],



Table I. About 93% of particulate PAH were 3 or 4 ring compounds, and the highest concentrations were analyzed for phenanthrene, fluoranthene and pyrene. Nine of particulate PAH consists of probably or possibly carcinogenic PAH compounds (glass 2A or 2B, according to IARC). The amounts of benzo[a]pyrene ranged from 38.4 ng to 79.5 ng/mg SOF between the particulate extracts. Comparisons of total PAH levels in all diesel extracts showed slightly higher concentrations for Set 1 than Set 2. The largest 20% difference in total PAH concentration between the two sets was detected for RD1 diesel extract, following with 7% for RD2 and EN97. The concentration of 1-nitropyrene was 26, 27.3 and 34 ng/mg SOF measured for RD2, RD1 and EN97 diesel particulate extract, respectively (Table I).

Table II shows the particulates (g/km), the sum of 14 PAH (μg/km) and the amount of SOF (mg/km) calculated in emission units. In comparison of reformulated RD2 and RD1 with standard fuel EN97, particulate emission decreased in two sets between 55 and 57%, and between 35 to 32%, respectively. The sum of the 14 particulate PAH in emission decreased between 64 and 70% for RD2 and between 35 and 45% for RD1 in comparison with EN97. The amount of SOF decreased between 58 and 60% for RD2 and 47% for RD1, when compared to EN97 (Table II).

## Mutagenicity by Ames test

After exposing *Salmonella typhimurium* test strains TA98 and YG1021 to diesel particulate extracts, the mutagenic activities were measured (Fig. 1). Strain YG1021 showed a 2-fold activity of PAH compared to strain TA98. In two sets, the mutagenic activity in strains TA98 and YG1021 was lower for reformulated diesel particulate extracts than in EN97. The decrease of mutagenicity in TA98 strain, when compared to EN97, was from 48 to 71% for RD2 and from 40 to 44% for RD1. In YG1021 strain, the decrease

TABLE II Amounts of particulates, total PAH concentrations and soluble organic fractions (SOF) emitted per kilometer of three Diesel fuels burned by the light-duty engine of the test vehicle. Average values were calculated from the number of tests performed on two sets of emission testing

| | *Set 1* | | | *Set 2* | | |
|---|---|---|---|---|---|---|
| | *RD2* | *RD1* | *EN97* | *RD2* | *RD1* | *EN97* |
| Number of tests | 24 | 20 | 17 | 11 | 7 | 4 |
| Particulates (g/km) | 0.045 | 0.065 | 0.100 | 0.040 | 0.064 | 0.094 |
| Sum of 14 PAH (μg/km) | 93 | 169 | 260 | 65 | 117 | 214 |
| SOF (mg/km) | 19 | 24 | 45 | 18 | 24 | 45 |

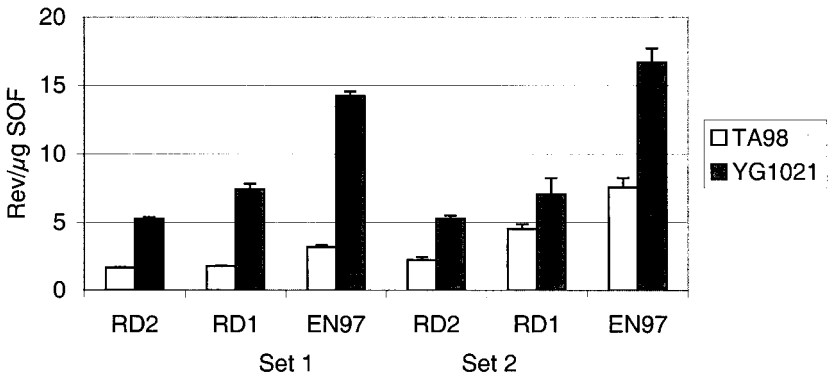


FIGURE 1 Mutagenic activity of Diesel particulate extracts (Rev/μg SOF) in *Salmonella* TA98 and YG1021 strains measured without external metabolic activation.



was from 63 to 69% for RD2 and from 48 to 58% for RD1, when compared to EN97. There was no correlation between the mutagenic activity and the 14 particulate PAH analyzed ($r=-0.033$, $p=0.950$, $n=6$ in strain TA98; $r=0.184$, $p=0.728$, $n=6$ in strain YG1021), but a strong correlation between the mutagenic activity and the 1-NP concentration ($r=0.996$, $p=0.059$, $n=3$ in strain TA98; $r=0.997$, $p=0.051$, $n=3$ in strain YG1021).

## DNA adduct studies *in vitro*

Figure 2 shows PAH-DNA adduct formation with [S9(+) and XO(+)] and without [S9(−) and XO(−)] the metabolic activation systems. Adduct levels were higher in all samples, when diesel extracts were incubated with the activation mixture [S9(+) and XO(+)]. Adduct levels did no differ clearly between these diesel extracts, when analyzed without the metabolic activation [S9(−) and XO(−)]. When the activation mixture was used, DNA adduct formation by EN97 was higher than that by two reformulated diesel extracts. All Diesel extracts formed more adducts by using the nitroreductive than the oxidative activation system. The 14 particulate PAH analyzed in extracts did not correlate with adducts formed by using the S9 activation mixture ($r=0.109$, $p=0.837$, $n=6$) or by using the xanthine oxidase ($r=0.268$, $p=0.608$, $n=6$). When the S9 activation of particulate extracts was used, the adduct levels of RD2 and RD1 were from 72 to 82% lower when compared with those of standard fuel EN97. Similar results

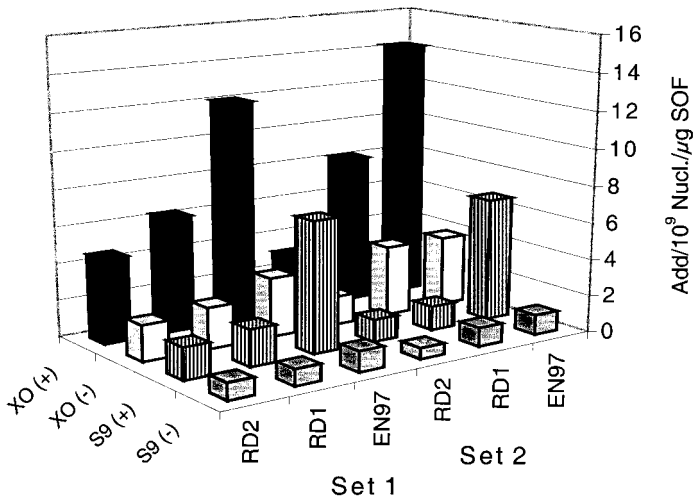


FIGURE 2 DNA adducts formed *in vitro* with calf thymus DNA by incubating Diesel particulate extracts with [S9(+), XO(+)] and without [S9(−), XO(−)] metabolic activation systems.



were obtained, when the XO activation was used, showing the reduction of adducts from 42 to 78%.

Figure 3A shows that the mutagenicity measured in YG1021 test strain was in good accordance with adduct levels analyzed with the corresponding activation system [XO(+)]. A better association between TA98 mutagenicity and DNA adducts (S9−) was obtained for Set 1 than for Set 2 (Fig. 3B). Overall results of mutagenicity and DNA adduct formation of this study showed that higher amounts of direct-acting PAH (nitro-PAH) than parent PAH requiring a metabolic activation was analyzed in diesel particulate extracts.

Correlation was better between the mutagenicity in strain YG1021 and DNA adducts formed by the nitroreductive enzyme [XO(+)] from PAH compounds ($r = 0.965$, $p = 0.002$, $n = 6$) than between the mutagenicity in TA98 and DNA adducts without the metabolic activation [S9(−)] ($r = 0.360$, $p = 0.483$, $n = 6$) (Fig. 4). Correlations obtained for the mutagenicity and DNA adducts were slightly better in Set 1 ($r = 0.992$, $p < 0.0001$, $n = 6$) than in Set 2 ($r = 0.888$, $p = 0.018$, $n = 6$).

## DISCUSSION

The mutagenicity and DNA adduct formation confirmed that diesel extracts contained both direct-acting and metabolically activated polyaromatic compounds. High mutagenicity measured in YG1021 showed that direct-acting nitro-PAH are more potent compounds in diesel emission than parent PAH. Furthermore, YG1021 strain is more sensitive than TA98 in measuring the effect of direct-acting PAH. This is in agreement with Arimochi's study[12], in which for strain YG1021 the higher mutagenicity of 1-nitropyrene, the nitroreductase activity and the DNA adduct level were measured, when compared to strains TA98 and TA98NR. In this study, no other nitro-PAH than 1-NP was detected, due to the methodological difficulties to measure low concentrations of pure compounds in complex mixtures. Comparisons of 1-NP levels with the

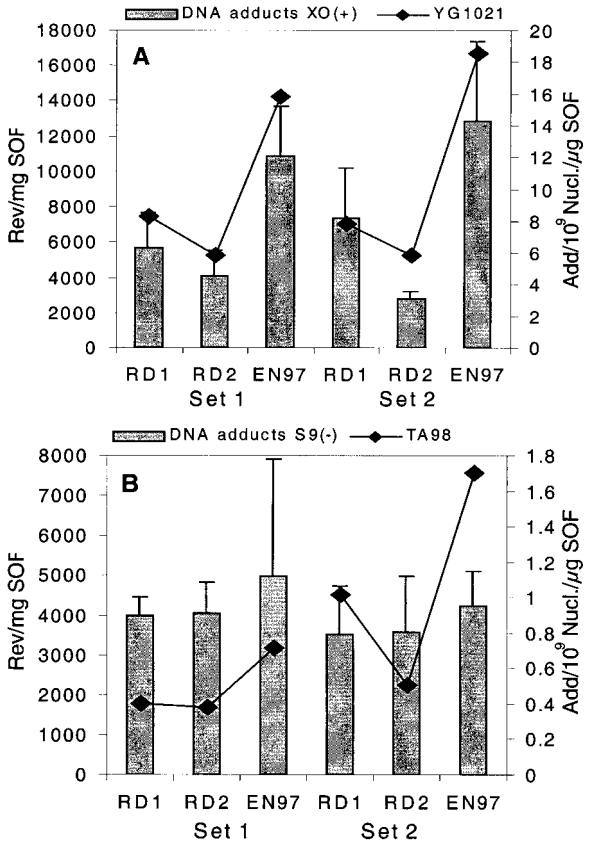


FIGURE 3 Comparison of DNA adduct formation by direct-acting PAHs and mutagenicity in YG1021 (A) and TA98 (B) strains after exposure to Diesel particulate extracts.



mutagenic activity tested, showed that the highest concentration of 1-NP in EN97 was in agreement with the highest mutagenicity measured in strain TA98 and YG1021 in both sets (Fig. 1). High mutagenicity and adduct formation measured in nitroreductive test system is in agreement with other studies[13–17].

We have earlier investigated the activation of PAH derived from Diesel particles in human skin tissue samples and in human biomonitoring study[18]. Lower level of DNA adducts were detected in human skin tissue cultures formed by reformulated Diesel extracts, when compared to those formed by standard reference Diesel fuel. Individual differences in PAH metabolism was detected by comparing lymphocyte DNA adducts of bus garage and waste collection workers with PAH concentrations measured from the personal air samples[18]. The specific harmful compound derived from Diesel emissions remains to be characterized. In this study, we were able to show the identification of 1-nitropyrene by GC/MS analysis. However, the present method did not resolve further information of low level nitro-PAH in diesel extracts. HPLC combined with a thin layer chromatography is a promising technique to identify adducts derived from PAH mixture[19].

In summary, reformulation decreased the amount of particulates and particulate PAH in diesel emission and, therefore, the genotoxicity of Diesel extracts.

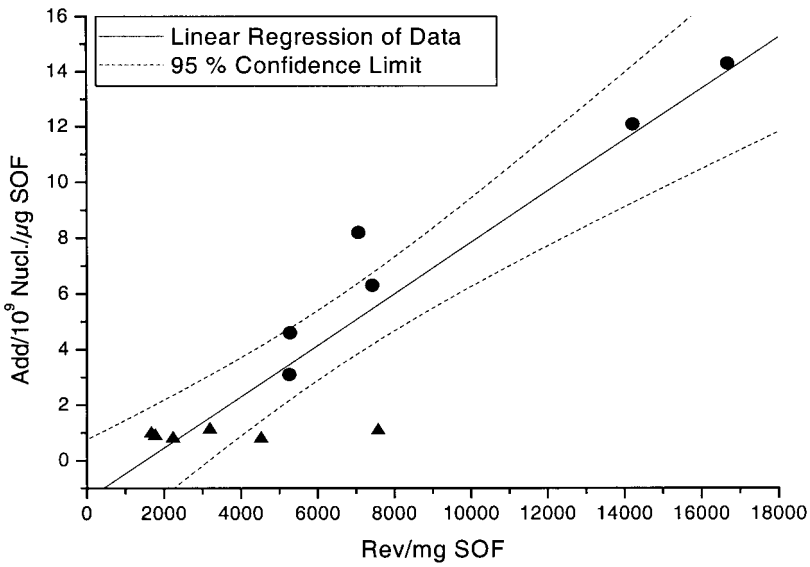


FIGURE 4 Correlation between DNA adducts formed *in vitro* with CT-DNA and mutagenicity in TA98 strain (▲) and in strain YG1021 (●), ($r = 0.922$, $p < 0.0001$, $n = 12$).



Reductions due to the fuel reformulation, when compared to European standard diesel fuel, was in particulate 32–57%, in particulate PAHs 35–70%, in mutagenicity 48–71% and in DNA adduct formation 42–82%. Particulate extract of RD1 contained higher concentrations of 14 PAH compounds than RD2, which may explain the difference in mutagenicity and *in vitro* CT DNA adduct levels between the reformulated diesel extracts. However, there are hundreds of other PAH compounds (than the 14 analyzed here) in Diesel particulate extracts that also influence the mutagenicity and DNA adduct formation. The reduction of mutagenicity and lower DNA adduct formation, compared to the standard European Diesel fuel, could partly be explained by differences in activation of PAH compounds in extracts. In clarifying cellular effects of Diesel exhaust, DNA adducts and Ames mutagenicity test is an applicable method of evaluating genotoxicity of Diesel particle derived PAH and nitro-PAH.

## *Acknowledgements*

This study was supported by Neste Oyj's Foundation, and Technology Development Center of Finland. Nina Tamminen from Finnish Institute of Occupational Health and the staff of Fortum Technology Centre are thanked for skillful technical assistance. Marcia Nishioka from Battelle Memorial Institute, Columbus, OH is thanked for the valuable analysis of nitro-PAH.

## *References*

[1] IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 46. International Agency for Research on Cancer (IARC), Lyon (1989).
[2] J.L. Durant, W.F. Busby Jr, A.L. Lafleur, B.W. Penman and C.L. Crespi, *Mutat. Res.*, **371**, 123–157 (1996).

[3] I.B. Weinstein, *Cancer Res*., **48**, 4135–4143 (1988).
[4] T. Kuljukka, K. Savela, K. Peltonen, S. Mikkonen and L. Rantanen, *SAE Technical Paper*, 982650 (1998).
[5] Concawe Report No. 6/97, Motor vehicle emission regulations and fuel specifications. Part 2. Detailed information and historic review (1970–1996) (1997).
[6] NIOSH Manual of Analytical Methods (NMAM), Polynuclear aromatic hydrocarbons by HPLC, Method 5506, Issue 3 (1998).
[7] M.G. Nishioka, C.C. Howard, D.A. Contons, L.M. Ball and J. Lewtas, *Environ. Sci. Technol*., **22**, 908–915 (1988).
[8] D.M. Maron and B.N. Ames, *Mutat. Res*., **113**, 173–215 (1983).
[9] J. Lewtas, R.L. Bradow, R.H. Jungers, B.D. Harris and R.B. Zweiinger, *Environ Int*., **5**, 383–387 (1981).
[10] R.C. Gupta, In: D.H. Phillips, M. Castegnaro, H. Bartsch (Eds.), *Postlabeling Methods for Detection of DNA Adducts*, pp. 11–23. International Agency for Research on Cancer (IARC), Lyon (1993).
[11] IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 32, Suppl. 7. International Agency for Research on Cancer (IARC), Lyon (1987).
[12] H. Arimochi, T. Kinouchi, K. Kataoka, T. Kuwahara and Y. Ohnishi, *J. Med. Invest*., **44**(3–4), 193–198 (1998).
[13] J. Gallagher, U. Heinrich, M. George, L. Hendee, D.H. Phillips and J. Lewtas *Carcinogenesis*, **12**, 1685–1691 (1991).
[14] P.P. Fu and D. Herreno-Saenz, *Environ. Carcino. Ecotox. Revs*, **C17**(1), 1–43 (1999).
[15] J. Lewtas and M.G. Nishioka, In: R.C. Howard, S.S. Hecht and F.A. Beland (Eds.), *Nitroarene Occurrences, Metabolism and Biological Impact*, pp. 61–72. Plenum Press, New York (1991).
[16] L. King, M.H. George, J.E. Gallagher and J. Lewtas, *Chem. Res. Toxicol*, **7**, 503–510 (1994).
[17] H.S. Rosenkranz, *Mutat. Res*., **367**, 65–72 (1996).
[18] T. Kuljukka-Rabb, L. Kuusimäki, M. Saarikko, K. Peltonen, L. Rantanen, S. Mikkonen and K. Savela, *Polycyclic Arom. Comp*., **21**, 273–285 (2000).
[19] K. Savela, L. King, J. Gallagher and J. Lewtas, *Carcinogenesis*, **16**, 2083–2089 (1995).