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Determination of in Vitro Biotoxicity in Exhaust Particulate Matter from Heavy-Duty Diesel Engine

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Diesel engines have found wide application in heavy duty vehicles and equipment for many years and in recent years have been used to an increasing extent in light-duty vehicles and cars. This latter usage is projected to increase substantially in the future, both in China and worldwide. Diesel fuels have close relationship with industry production and other human activities, but they also result in large amount of pollutants that are released into the environment. The emission of diesel fuel combustion is a complex mixture of particulate and vapor-phase compounds. The soluble organic fraction (SOF) from diesel exhaust particles (DEP) contains thousands of organic constituents and is thought to contain the compounds of most environmental significance, including a variety of polycyclic aromatic hydrocarbons (PAHs) , dioxin and so on (Shinji et al., 2002). In recent years, increased concern has developed for the potential health effects of DEP. It is necessary to determine whether exposure to DEP at environmentally relevant concentration poses a threat to human and to the ecosystem as a whole.

In this reseach, we evaluated the estrogenic, genotoxic and mutagenic effects of SOF of diesel exhaust. The assay battery included:(a)Estrogenic activity detected by a recombinant bioassay; (b)*Salmonella typhimurium*, TA98 and TA100 strains, with and without liver fraction (S9mix);(c)Comet assay on rat fibrocytes (Singh et al.,1998). The comet assay, i.e., single cell gel electrophoresis (SCGE), is becoming a major tool in environmental pollutant biomonitoring, both in vivo and in vitro (Speit et al.,1996).The aim of our study was: (a) to evaluate the estrogenic activities in the SOF from DEP with the above mentioned recombinant yeast bioassay; (b) to detect the genotoxicity of the sub-fractions on various genetic targets, assess the suitability of SCGE on rat fibrocytes for diesel exhaust pollution monitoring, together with standard short-term mutagenicity tests.

MATERIALS AND METHODS

DEP, sampled from a dilution tunnel manufactured by Horiba Company, was harvested from the diesel exhaust of CY6102BZLQ-type engine produced by

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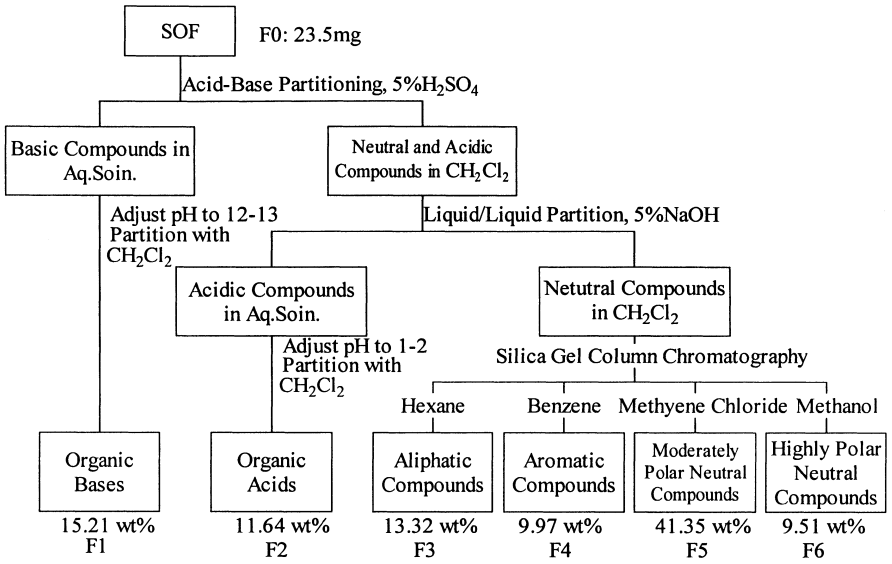

Figure 1. Scheme of the fractionation procedure

Dongfeng Chaoyang Diesel Engines Company Ltd, China and collected with the ECE R49-13 test mode. We repeated this process for 20 times and collected 57.1mg DEP. The DEP were kept in a sealed bottle at -20°C in the dark.

For this study the SOF were extracted using the Soxhlet technique under yellow fluorescent lights. All solvents used in this study were Burdick and Jackson distilled-in-glass quality. The DEP was extracted with 20mL of methylene chloride for 24h. The resulting extract, including SOF and solvent, was concentrated to 1 mL by rotary film and vortex evaporation prior to separation. The sample extracts were removed for replicate fractionation and bioassay analyses. The SOF (F0) we obtained was 23.5mg. A brief description of the fractionation diagram and the proportion of the each fraction to the tatal SOF are given in Fig 1(Joellen et al.,1990).

Chemical analysis of sub-fraction samples were performed by gas chromatography-mass spectrometry using a Hewlett-Packard 5890 Series Π gas chromatograph and a Hewlett-Packard 5971 Mass Selective Detector. The organic compounds identified in F3, F4 and polar fraction (F5 and F6) has been made and are listed in Table 1. Meanwhile, we didn't analyze the organic bases and the organic acids because of their higher bioling point, which should be made a further research.

The estrogen bioassay employed in this study involves the use of a genetically modified yeast strain to determine the ability of a test agent to trans-activate the estrogen receptor. This yeast strain contains a human estrogen receptor gene and a

Table 1. The organic compounds identified in F3, F4 and polar fraction (F5 and F6)

Aliphatic fraction	Aromatic fraction	Polar fraction
Octadecane; Nonadecane; Elcosane; Henelcosane.	Naphthalene; Fluoranthene; Acenaphthylene; Acenaphthene; Chrysene; Fluorene; Anthracene; Phenanthrene; Pyrene; Benzo(a)Anthracene; Benzo(a)Pyrene; Benzo(b)Fluoranthene; Benzo(g,h,i)Perylene; Dibenz(a,h)Anthracene; Indeno(1,2,3-cd)Pyrene.	Hexanedioic acid Bis (2-methylpropyl) ester; Butanedioic acid Bis (2-methylpropyl) ester; 1,2-Benzenedicarboxylic Bis (2-methylpropyl) ester; 1,2-Benzenedicarboxylic Butyl 2-methylpropyl; Hexanedioic acid Bis (2-ethylhexyl) ester; 1,2-Benzenedicarboxylic Diisootyl ester; Dibutyl phthalate.

reporter gene coding for the β-galactosidase. Test agents with estrogenic activity activate the receptor gene in yeast strain and subsequently result in the production β-galactosidase. The estrogenic activity of chemicals is quantified by determining the activity of the β-galactosidase (Routledge and Sumpter, 1996). The calculation relating to estrogenic activity poceeded following the prescription of Klaus et al. (1999). EC_{50} values were calculated from dose response curves obtained by fitting the data by following equation using the least squares method: Cs [μm] = 10^6 (Ex_S - Ex_B)/(ε_N×d), where Ex_S is the Ex_{420nm} of the enzyme reaction supernant of the sample, Ex_B the Ex_{420nm} of the enzyme reaction supernant of the blank, ε_N the ε for *o*NPG in the enzyme assay reaction mix (4.666×10^3 cm^2/mole) and d the diameter of the cuvette (1cm). The estrogenic relative potency (RP) of the test samples was computed by dividing the concentration of 17 β-estradiol (E2) giving 50% induction of β-galactosidase activity (EC_{50}) by the EC_{50} of the test samples, and then multiplying these values by 100. The RP value for E2 was 100 by definition. The relative inductive efficiency (RIE) was the ratio between maximal β-galactosidase activity achieved with the test sample and that of E2 multiplied by 100. By definition, E2 had an RIE of 100.

Mutagenicity assays were performed according to the method of Maron and Ames (1983) modified by De Meo et al (1996). The multagenic activity of the sub-fractions of the SOF was studied using *S. typhimurium*, strains TA98 and TA100, with or without microsomal activation. The strains were kindly provided by Tianjin Sanitation and Disease Prevention Center.

The Comet assay in this study used the rat fibrocytes. The assay was performed according to Singh et al (1998). The image analysis system allows us to make a quantitative description of the comet using various parameters such as the tail moment (TM), which is an integrated value considering both the distance and the

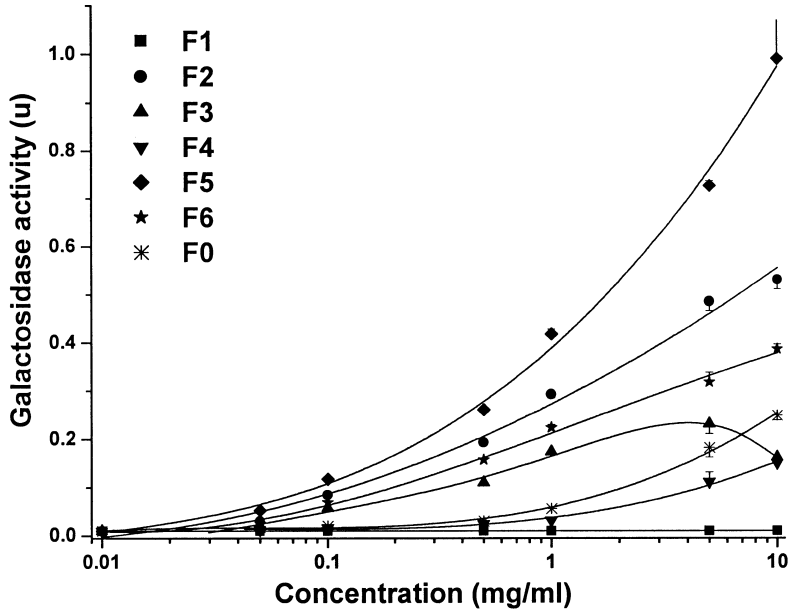

Figure 2. The estrogenic activity of assay samples from diesel.

amount of migrated DNA, i.e., tall length × %DNA fluorescence intensity in tail. The comet parameter TM was chosen to represent the data on genotoxic effects.

RESULTS AND DISCUSSION

Each yeast assay included a positive dose-response curve of 17 β-estradiol (E2) as a reference (Jingxian et al., 2003). Calibration curves of estrogen were produced by analysis of standards 0.01-100 ng/ml for E2 in selective media for estrogenic activity. SOF (F0) were fractionationed into six parts: F1-F6. The estrogenic activities of SOF and six sub-fractions were determined by yeast bioassay. Fig 2 and Table 2 showed the dose-response relationship of estrogenic activity and estrogenic relative potency of sub-fractions from diesel exhaust particles. For each yeast assay, the response to a test sample was judged as fully estrogenic if the RIE was >75%, partially estrogenic if the response was 25-75%, weakly estrogenic if the response was 10-25%, and negative if the response was below 10% of the response induced by 17 β-estradiol (Andersen et al., 1999). According to Fig 2 and Table 2, the samples with highly estrogenic relative inductive efficiency in relation to the positive were the F2 and F5 that was 29.8% and 55.2%, respectively, both partially estrogenic. The main compounds in F2 might be acids and phenols. And the main compounds in F5 might be substituted phenols and big molecular alcohols (Jingxian et al., 2003). These

Table 2. RP and RIE of DEF and their sub-fractions in response to recombinant yeast bioassay.

Samples	EC_{50} (mg/ml)	RIE	RP×10^{-6}
F0	3.2	13.3	4.5
F1	NT	NT	NT
F2	0.79	29.8	18.2
F3	0.68	15.5	21.2
F4	2.8	7.7	5.1
F5	2.1	55.2	6.9
F6	0.80	21.0	18.0

Abbreviations: RIE indicates the ratio between maximal β-galactosidase activity achieved with the assay sample and that of E2×100. RP is computed by compared to E2 (100) by 10^{-6}mg/ml determined with the recombinant yeast cell bioassay. NT=not tested. F1 was not detected because of its exceeding the detection limit.

major compounds are the likely candidates responsible for the partially estrogenic activities of F5. On the other hand, most assay samples were weakly estrogenic and their emission contain compounds such as aromatic, carbonyls, aromatic alcohols, substituted phenols, PAHs and derivatives. Studies on the relationships between chemical structures and estrogenic activities have suggested that most chemicals with estrogenic activities contain phenolic groups (Schultz et al., 2000). Consequently, aromatic carbonyl, aromatic alcohol, substituted phenols and some PAH derivatives, etc. in the emissions are likely responsible for the estrogenic activities observed. The main compounds in the aromatic fraction F4 that responsible for the very weak estrogenic activity might be PAHs and derivatives (Jingxian et al., 2003). PAHs have been considered as possible endocrine disrupter. But in this study, PAHs showed weakly estrogenic inductive efficiency. This may be attributed to weaker binding to ER by PAH compounds or to the presence of AhR agonists in the samples. These factors could suppress E2 activity in vivo (Fielden et al., 2000). This possibility needs to be verified experimentally. In recent years, growing attention has been focused on the potential synergistic effect of natural and synthetic compounds. It seems that some compounds existing in F5 may have antagonistic effect on part F3 and F4 or have toxic effect on yeast cells. The yeast bioassays employed cannot discriminate between estrogenic and anti-estrogenic compounds. Because of the limitation of the space, the quantitative side of each assay is not specified in the paragraphs but is listed in the figure and table.

Table 3 shows the mutagenicity of the assay samples. Some sub-fractions might react differently with or without S9 in the same strain. We used the TA98-S9 and TA100-S9 to detect the direct carcinogens, whereas we used the TA98+S9 and TA100+S9 to test the indirect mutagens. For example, F1 was the most mutagenic of the seven sub-fractions in TA100+S9, so it indicated that organic bases compounds whereas other assay samples contained higher amounts of indirect acting compounds in

Table 3. Mutagenic activities of the seven assay samples using the Ames test[a].

Sample	Dose (mg/plate)	Number of revertants			
		TA98–S9	TA98+S9	TA100–S9	TA100+S9
F0	0.25	7±1	37±4**	249±15**	211±12**
	0.5	21±1	46±5**	198±12**	198±10**
	1	52±2**	62±8**	331±11**	189±9**
F1	0.25	134±9	44±5	266±18	289±14**
	0.5	188±7**	113±4	253±12	346±26**
	1	231±16	NT	550±31	792±35**
F2	0.25	27±2**	24±2**	128±21**	209±15**
	0.5	24±1**	29±1**	123±18**	133±13**
	1	21±2**	31±3**	126±11**	144±13**
F3	0.25	13±1	25±2	98±12	156±12**
	0.5	15±0	23±2	185±15**	140±9**
	1	16±1	24±3	158±12	134±12*
F4	0.25	75±2**	139±11**	241±16**	510±46**
	0.5	107±4**	159±21**	581±24**	531±51**
	1	139±4**	201±15**	594±26**	549±47**
F5	0.25	25±2*	31±3	126±10	159±14**
	0.5	62±2**	79±7**	127±11	244±17**
	1	79±3**	91±5**	244±18**	256±15**
F6	0.25	21±2	39±4**	142±9	156±11**
	0.5	34±5*	61±5**	221±16**	187±12**
	1	42±6**	67±6**	207±15**	260±18**
Spontaneous revertants		20	25	144	96

[a]Mutagenity is expressed as the mean number of revertants/plate found on the two strains of *S. typhimurium* ± SEM for two independent experiments. NT – not tested. Effects are considered significantly positive with respect to spontaneous revertants (t–test): *P<0.05; **P<0.01.

TA100+S9; for F4 in TA100-S9, it contains more direct acting compounds. For F0, the organic bases compounds show a more effect in TA98+S9, TA100-S9, and TA100+S9 (P<0.01, t-test), whereas the effect in TA98-S9 is not visible except that at the concentration of 1mg/plate. F1 and F3 is more potent in TA100+S9 (P<0.01, t-test) than in other three strains. The effect of F2 and F4 in TA98 and TA100 with or without S9 mix was more mutagenic than those of spontaneous revertants (P<0.01, t-test), so the mutagenicity of organic acids and aromatic compounds was strong with obvious dose-response relations, but organic acids in TA98+S9 with the dose of 1mg/plate suppressed the mutagenicity because of the higher concentration. F2 and F4 showed strong mutagenicity at all assay doses. For F0, F5 and F6, the polar compounds had positive results and some dose-response relations at high concentrations and vice versa at low concentrations. On other hand, some data

cannot be clearly interpreted. There is a great difference between the mutagenic activity of aliphatic compounds in the two strains. In this case, toxicity cannot be used to explain the different behaviors of the two strains. Some compounds might react differently in the two strain. Furthermore, some unknown compounds, or some interaction among mixture compounds, could have different mutagenic potency on the two strain.

The data from the comet assay (Table 4) indicate a higher genotoxic potency of all the assay samples at high doses compared to those at lower doses ($P<0.05$, t-test) according to their significant increase of tail moment values. For a relatively low dose, F2, F3, F4, F5 and F6 induces a significantly higher effect than the other assay samples ($P<0.05$, t-test), so they showed more genotoxicity than F0 and F1.

Table 4. Genotoxic activity of Comet assay samples on rat fibrocytes using the mean value of TM (μm). Effects are considered significantly positive with respect to untreated group (t-test).*$P<0.05$. Negative control – DMSO. Positive control – Melphalan.

dose Sample	50μl (1mg/ml)	25μl (1mg/ml)	12.5μl (1mg/ml)
F1	24.18*	13.54*	11.12
F2	22.11*	13.57*	11.78*
F3	30.61*	18.85*	13.50*
F4	30.23*	16.19*	13.58*
F5	24.14*	13.52*	12.54*
F6	21.81*	14.86*	11.87*
F0	19.81*	12.88*	11.45
Negative control		10.39	
Positive control		34.47	

A wide range of chemicals are present in the DEP and may pose a significant health risk for human populations. In this context, we evaluated the possible differences of estrogenic activities, mutagenicity and genotoxicity deriving from different assay samples by extracting organic materials. Both organic acids and polar neutral compounds seem more similar estrogenic/mutagenic/genotoxic responses in the three test systems. The different responses of the various genetic targets suggest a wide variability in the chemical composition of the tested samples. This depends on both the quantitative and qualitative differences between the sub-fractions analyzed. Our results indicate that, in all the test systems applied, genotoxic activities are widely affected by the assay samples. DNA damage on rat fibrocytes shows a wide sensitivity. Furthermore, we found a high correlation of comet results with *Salmonella*, in agreement with previous reports (Anisimov et al., 2000) for which the correlation was dependent on action mechanisms of the analyzed samples.

Our results state that special care is needed in monitoring a complex mixture such as DEP. Differences of DNA-effective compounds concentration were found in different sub-fractions, probably in relation to the different formation mechanisms, chemical composition, and sources. Furthermore, the wide variability of test response indicates that compound genotoxicity is detected with varying sensitivity in each assay, which is probably due to innate differences in the cells, different mechanisms expressing the effects, and differences in test conditions. The data demonstrate the limitations in predicting estrogenic and genotoxic potential of environmental mixtures such as DEP based on only one biological system. The three biological systems we used appear to be sensitive and be able to monitor the pollution arising from DEP. Concerning the comet assay, it seems to be a sensitive genotoxicity test for diesel exhaust mixtures. But because of wide variability between test results in the different assays, it is not possible to evaluate whether this test system is more sensitive/specific than other test systems.

The emission from diesel fuel combustion is one of the most important pollution sources because of the large volume and widespread nature of diesel fuel combustion waste. Although the impact of estrogenicity, mutagenicity and genotoxicity of these compounds on ecosystem and human health is unknown, the phenomena of their activities from prominent pollution sources demands further research about their potential to cause adverse effects.

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