

## Strong mutagenic effects of diesel engine emissions using vegetable oil as fuel

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**Abstract** Diesel engine emissions (DEE) are classified as probably carcinogenic to humans. In recent years every effort was made to reduce DEE and their content of carcinogenic and mutagenic polycyclic aromatic compounds. Since 1995 we observed an appreciable reduction of mutagenicity of DEE driven by reformulated or newly designed fuels in several studies. Recently, the use of rapeseed oil as fuel for diesel engines is rapidly growing among German transportation businesses and agriculture due to economic reasons. We compared the mutagenic effects of DEE from two different batches of rapeseed oil (RSO) with rapeseed methyl ester (RME, biodiesel), natural gas derived synthetic fuel (gas-to-liquid, GTL), and a reference diesel fuel (DF). The test engine was a heavy-duty truck diesel running the European Stationary Cycle. Particulate matter from the exhaust was sampled onto PTFE-coated glass fibre filters and extracted with dichlo-

romethane in a soxhlet apparatus. The gas phase constituents were sampled as condensates. The mutagenicity of the particle extracts and the condensates was tested using the *Salmonella typhimurium*/mammalian microsome assay with tester strains TA98 and TA100. Compared to DF the two RSO qualities significantly increased the mutagenic effects of the particle extracts by factors of 9.7 up to 59 in tester strain TA98 and of 5.4 up to 22.3 in tester strain TA100, respectively. The condensates of the RSO fuels caused an up to factor 13.5 stronger mutagenicity than the reference fuel. RME extracts had a moderate but significant higher mutagenic response in assays of TA98 with metabolic activation and TA100 without metabolic activation. GTL samples did not differ significantly from DF. In conclusion, the strong increase of mutagenicity using RSO as diesel fuel compared to the reference DF and other fuels causes deep concern on future usage of this biologic resource as a replacement of established diesel fuels.

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mammalian microsome assay

### Abbreviations

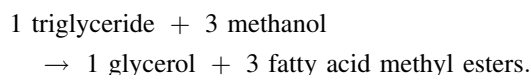
DCM	Dichloromethane
DEE	Diesel engine emissions
DEP	Diesel engine particles
DF	Diesel fuel
DMSO	Dimethyl sulfoxide
ESC	European Stationary Cycle
GHG	Atmospheric greenhouse gas
GTL	Gas to Liquid fuel
FAME	Fatty acid methyl esters
PAC	Polycyclic aromatic compounds

mRSO	Preheated rapeseed oil
PTFE	Polytetrafluoroethylene
RME	Rapeseed methyl esters
SME	Soybean methyl esters
RSO	Rapeseed oil

## Introduction

The replacement of petroleum-derived fuels by biofuels from renewable resources has gained worldwide interest and is scientifically investigated for its environmental costs and benefits (Hill et al. 2006; Ragauskas et al. 2006). Especially the reduction of atmospheric greenhouse gas (GHG) is covered by recent discussions, since the combustion of vegetable-oil derived fuels instead of fossil fuel reduces net GHG emissions (Koonin 2006). Less attention has been paid to the possible hazards for human health (Krahl et al. 2001; Swanson et al. 2007).

Fatty acid methyl esters (FAME) are proven as a suitable alternative to fossil diesel fuel (DF) producing similar or even lower emissions (Krahl et al. 1996; Bagley et al. 1998; Bünger et al. 2000a). They are called biodiesel and can be produced from different oil plants, e.g., rapeseed (canola), oil palm, soybean, and sunflower. Biodiesel is produced by transesterification of triglycerides from vegetable oils with methanol (Krahl et al. 1996), resulting in a fuel with similar properties as mineral oil derived fuels. The following equation generally shows the transesterification of triglycerides to the corresponding methyl esters:



Diesel engine emissions (DEE) contain mutagenic and carcinogenic polycyclic aromatic compounds (PAC) on the surface of the emitted particles and—to a lesser amount—in the gaseous phase (Schepers and Bos 1992). The formation of PAC depends on type of engine, engine load, fuel properties, and effectiveness of exhaust after treatment. In previous studies, we demonstrated the influence of different fuels—including rapeseed methyl ester (RME) and soybean methyl ester (SME)—on the mutagenic activity of DEE (Bünger et al. 1998; Bünger et al. 2000b; Bünger et al. 2006).

Recently, the use of rapeseed oil as fuel is emerging especially in the transportation sector for economic reasons. In this investigation, two batches of rapeseed oil—a cold pressed rapeseed oil (RSO) and a rapeseed oil with lowered viscosity and fuel preheating in the tank (mRSO)—were compared with a rapeseed-oil derived

biodiesel (rapeseed methyl ester, RME), a natural-gas derived synthetic fuel (gas to liquid, GTL), and a common diesel fuel (DF, reference fuel) for their influence on the mutagenicity of resulting diesel engine emissions (DEE).

## Materials and methods

### Fuels and chemicals

The reference DF, which met the EU-standard EN590, was delivered by Haltermann Products, Hamburg, Germany, GTL by Shell AG, Hamburg, and RME by ADM Oelmühle, Hamburg, Germany. Rapeseed oils were commercially offered as truck biofuel and purchased from two German providers.

Nutrient media and most chemicals for the mutagenicity test system were obtained from Difco Laboratories (Detroit, USA) and Sigma (Deisenhofen, Germany). Methyl methanesulfonate [CAS 66-27-3], 2-aminofluorene [CAS 153-78-6], and -naphthoflavone [CAS 6051-87-2] were obtained from Aldrich (Milwaukee, USA), phenobarbital [CAS 50-06-6] from Sigma (Deisenhofen, Germany). Dichloromethane (DCM) and dimethyl sulfoxide (DMSO), spectrometric grade, was provided by Merck (Darmstadt, Germany).

### Engine test procedures and sampling method

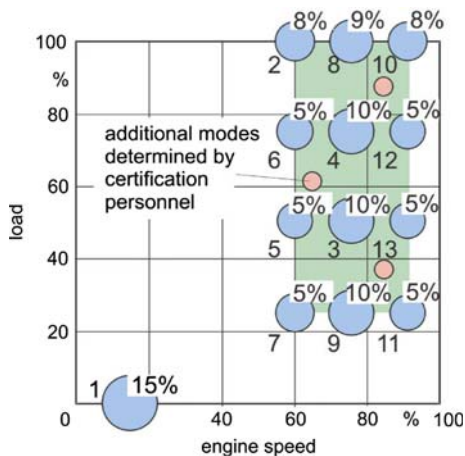
The investigations were carried out at the emission test facility of the Institute for Technology and Biosystems Engineering at the German Agricultural Research Centre in Braunschweig, using a Mercedes-Benz engine OM 906 LA with turbocharger and intercooler meeting the Euro 3 exhaust limits (Table 1).

Exact engine load during test runs was accomplished by coupling the crankshaft to a Froude Consine eddy-current brake. Engine test runs were in accordance with the 13-mode European Stationary Cycle (ESC, Fig. 1), which does not include a cold start phase. For the test series with mRSO, the fuel was preheated to 70°C as it is carried out by several commercial two-tank system conversion kits for diesel trucks running regularly on vegetable oil. The small second tank is needed for diesel fuel, which is combusted only during the cold start phase and immediately before stopping the engine.

Particulate matter of each test cycle was collected from the exhaust stream onto one glass fibre filter coated with PTFE (Teflon) (T60 A20, Pallflex Products Corp., Putnam, CT, USA). The gas phase constituents were sampled as condensates using an intensive cooler (Schott, Germany). Condensed compounds were desorbed from the cooler with 100 ml DCM.

**Table 1** Technical data of Mercedes-Benz engine OM 906 LA

Piston stroke	130 mm
Bore of cylinder	102 mm
Number of cylinders	6
Stroke volume	6,370 cm <sup>3</sup>
Rated speed	2,300 min <sup>-1</sup>
Rated power	205 kW
Maximum torque	1,100 Nm at 1,300 min <sup>-1</sup>
Compression ratio	17.4

**Fig. 1** Modes of the ESC test

Each fuel was tested three times, resulting in 15 particle filters and 15 condensates. The filters were conditioned (20°C, rel. humidity 50%), weighed before and after sampling to determine the total particulate matter, and stored at -18°C. Extraction of the soluble organic fraction from the filters was performed with 150 ml DCM in a soxhlet apparatus (Brand, Wertheim, Germany) for 12 h in the dark (cycle time 20 min.). The extracts as well as the condensates were reduced by rotary evaporation (Heidolph, Kehlheim, Germany) and dried under a stream of nitrogen. They were redissolved in 4 ml DMSO immediately before use.

#### Mutagenicity assay

This study employed the revised standard test protocol of the *Salmonella typhimurium*/mammalian microsome assay that detects mutagenic properties of single compounds as well as of complex mixtures by reverse mutation of a series of *Salmonella typhimurium* tester strains, bearing mutations in the histidine operon (Maron and Ames 1983). Tester strains TA98 and TA100 were used, detecting mutagens that cause frameshift mutations and base-pair substitutions. These strains were shown to be most

sensitive to mutagens of organic extracts of diesel engine particles (DEP) (Clark and Vigil 1980; Claxton 1983).

Tests were performed with and without metabolic activation by microsomal mixed-function oxidase systems (S9 fraction). Preparation of the liver S9 fraction from male Wistar rats was carried out as described by Maron and Ames (1983). Phenobarbital and  $\beta$ -naphthoflavone (5,6-benzoflavone) were used for induction of liver enzymes. These substances were proven to be safe and adequate substitutes for Arochlor 1254 (Matsushima et al. 1976). The mutagens methyl methanesulfonate (10  $\mu$ g/ml in distilled water) and 2-aminofluorene (100  $\mu$ g/ml in DMSO) were used as positive controls.

Immediately before use, the extracts were dissolved in 4 ml DMSO and the following log 2 dilutions were tested: 1.0, 0.5, 0.25, and 0.125. Each concentration was tested both with and without 4% S9 Mix. Every extract was tested in triplicate. Plates were incubated at 37°C for 48 h in the dark, and revertant colonies on the plates were counted using an electronically supported colony counting system (Cardinal, Perceptive Instruments, Haverhill, Great Britain). The bacterial background lawn was regularly checked by microscopy, as high doses of the extracts proved toxic to the tester strains, resulting in a thinning out of the background.

#### Evaluation of results and statistical analysis

Mutagenic response was classified positive if a reproducible, dose-dependent increase of the number of revertant colonies was observed (Mortelmans and Zeiger 2000). Revertant numbers of the positive results (mean  $\pm$  standard deviations) were estimated from the initial linear part of the dose-response curves. Differences between the fuels were tested for significance using Student's *t*-test for independent variables, two-sided, using StatView for Windows, Version 4.57, Abacus Concepts Inc., Berkeley, CA, USA.

#### Results

The results of the mutagenicity assays are shown in Table 2. Spontaneous reverse mutation frequency of TA98 was  $22 \pm 7$  and of TA100  $119 \pm 26$ , respectively. The mRSO—extract produced the highest number of revertant colonies in both tester strains with and without metabolic activation ( $\pm$ S9), reaching a nearly 60-fold increase in TA98 with metabolic activation.

The RSO—extract also induced a strong increase of mutations, whereas the exhaust extracts of DF, RME, and GTL caused minor mutagenic effects up to a maximum of tripling the spontaneous frequency of revertants. Compared with the reference DF, the two RSO qualities significantly

**Table 2** Numbers of revertants in tester strain TA98 and TA100 (mean  $\pm$  standard deviations of quadruple tests) induced by particle extracts and the corresponding exhaust condensates per L exhaust

	TA98 – S9	TA98 + S9	TA100 – S9	TA100 + S9
Particle extracts				
DF	51 $\pm$ 7	24 $\pm$ 6	106 $\pm$ 20	50 $\pm$ 18
GTL	46 $\pm$ 10	27 $\pm$ 7	74 $\pm$ 17	31 $\pm$ 16
RME	57 $\pm$ 13	**51 $\pm$ 6	*167 $\pm$ 24	62 $\pm$ 15
RSO	**494 $\pm$ 62	***422 $\pm$ 21	***576 $\pm$ 55	**321 $\pm$ 65
mRSO	***1,374 $\pm$ 74	***1,419 $\pm$ 153	***1,382 $\pm$ 69	***1,115 $\pm$ 108
Condensates				
DF	48 $\pm$ 16	36 $\pm$ 8	158 $\pm$ 43	96 $\pm$ 26
GTL	45 $\pm$ 16	23 $\pm$ 4	102 $\pm$ 51	88 $\pm$ 23
RME	35 $\pm$ 18	35 $\pm$ 21	119 $\pm$ 21	63 $\pm$ 9
RSO	*145 $\pm$ 30	**89 $\pm$ 11	266 $\pm$ 52	123 $\pm$ 33
mRSO	**646 $\pm$ 75	***465 $\pm$ 81	***814 $\pm$ 56	***568 $\pm$ 81

DF diesel fuel, GTL natural gas derived fuel, RME rapeseed methyl ester, RSO rapeseed oil, mRSO preheated rapeseed oil

Significance of differences of GTL, RME, RSO, and mRSO were tested versus common DF using Student's *t*-test for independent variables, two-sided, \*  $P < 0.01$ , \*\*  $P < 0.001$ , \*\*\*  $P < 0.0001$

increased the mutagenic effects of the particle extracts by factors of 9.7 up to 59 in tester strain TA98 and of 5.4 up to 22.3 in tester strain TA100. RME had a slightly higher mutagenic response in assays of TA98 with metabolic activation and TA100 without metabolic activation.

Compared with condensates of the other fuels, the RSO fuels caused stronger mutagenicity. As observed in assays with the extracts, condensates of RSO and mRSO showed a strong mutagenic response up to factor 13.5 than the reference fuel DF.

In parallel, the EU-regulated exhaust emissions were determined. With exception of NO<sub>x</sub> for RME, RSO and mRSO all results were within the Euro 3 limits. The bio-fuels led to an NO<sub>x</sub> increase of approximately 15–25% over the Euro 3 limit.

## Discussion

A decade ago, RME and SME were shown to be technically and environmentally adequate substitutes for fossil diesel fuel including a similar or even lower mutagenicity of their particle extracts (Krahl et al. 1996; Bagley et al. 1998; Bunger et al. 1998, 2000a).

Due to the fact that the production of RME from RSO is expensive, increasing effort is recently put into the introduction of RSO itself as a diesel fuel. Due to their chemical structure, these triglycerides have a substantially higher viscosity compared with FAME and petrol diesel fuels. It was the intention of this study to investigate how effective these molecules are combusted in a modern diesel engine.

Whereas the legally limited emissions like carbon monoxide, hydrocarbons, particulate matter, and nitrogen oxides of RSO differed from the other fuels tested only in acceptable margins, the mutagenic effects were unexpectedly strong.

The first hypothesis to explain this increase of the mutagenicity using RSO as fuel was the higher viscosity compared to RME and the other fuels. To test this hypothesis, a second RSO batch (mRSO) with reduced viscosity was investigated which was preheated to as it is carried out by several commercial available conversion kits according to the so called two tank solution. However, this modification of the fuel properties resulted in an even stronger mutagenicity of mRSO compared to RSO. At this point of our investigations we have no sound explanation for this effect but two hypotheses.

Possibly, the lowered viscosity advances the spray behaviour of the fuel in the combustion chamber, leads thereby to smaller droplets, and increases the total surface that can be considered by itself as phase interface between liquid and gas phase—and possibly as a reactive zone for the formation of mutagenic substances, e.g., PAC. The second hypothesis deals with the different properties of RME and RSO when heated and compressed in the combustion chamber. Triglycerides boil under decomposition—and those products are usually considered as hazardous to human health. In case the decomposition during the phase interface process increases the formation of mutagenic substances, a variation of the physical properties of the vegetable oil—if possible—may be helpful solving this problem.

In conclusion, this study demonstrates a very strong mutagenicity of DEP extracts and condensates from combustion of RSO and mRSO in the *Salmonella typhimurium*/mammalian microsome assay. This effect does obviously not depend on the higher viscosity than RME and the other fuels. Compared to modern fossil fuels (DF, GTL), biofuels can produce similarly low emissions of mutagenic compounds (RME) but may also have strong contrary effects (RSO, mRSO). In general, a systematic research concerning the influence of fuels on the exhaust composition of diesel (and gasoline) engines is urgently needed in order to develop fuels with lower emissions of hazardous substances.

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