Chemosphere 144 (2016) 1142–1147

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Synergistic androgenic effect of a petroleum product caused by the joint action of at least three different types of compounds

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HIGHLIGHTS

- An oil being synergistic in an androgenic assay was fractionated into SARA fractions.
- Synergism was lost when dosing separate fractions but combining fractions restored it.
- Synergism required testosterone, saturates and resins; thus min 3 different chemicals.
- Detailed chemical analyses could not reveal the identity of the causative compounds.

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 15 August 2015
Accepted 25 September 2015
Available online 23 October 2015

Handling editor: Shane A. Snyder

Keywords:
Synergism
Mixture toxicity
Yeast
Androgen receptor
Petroleum
SARA

ABSTRACT

In a previous study, we found a dose-dependent synergistic effect in recombinant yeast stably transfected with the human androgen receptor (AR), in response to co-exposure to testosterone and a commercially-available lubricant (engine) oil for cars. As there is relatively little knowledge on synergistic toxic effects and causative compounds, particularly for the androgenic system, the objective of the present study was to investigate this oil in more detail. The oil was fractionated into SARA fractions (so-called 'saturates', 'aromatics', 'resins', and 'asphaltenes') by open column chromatography. Surprisingly, when exposing the recombinant AR yeast to testosterone in combination with the separate SARA fractions, the synergistic effect could not be reproduced fully. After pooling the fractions again however, the full synergism returned. From subsequent exposures to combinations of two or three SARA fractions, it appeared that both the 'saturates' and the 'resins' fraction were required for obtaining the synergistic response with testosterone. This clearly demonstrates a synergistic effect related to the androgenic system caused by the joint action of at least three chemically-distinct compounds, or groups of compounds (i.e. testosterone, 'resins' and 'saturates'). Although detailed chemical analyses could not reveal the identity of the causative compounds and the in vivo relevance of the present results remains unclear, the results do add to the growing body of evidence on the potentially extremely complex character of mixture effects.

1. Introduction

Although humans and wildlife are exposed to complex mixtures of chemicals, current risk assessment practice is based on individual chemicals. Generally, exposure concentrations of single chemicals are compared to safe thresholds of the respective compounds, with the most sophisticated approach being the application of concentration addition or independent action concepts, i.e., the assumption that effects of separate chemicals can be added up (Backhaus et al., 2013). It is well-known however, that mixture toxicity caused by interactive effects of multiple chemicals may potentially occur. For example, numerous cases of antagonism exist in the toxicological literature, describing a joint effect of chemicals

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http://dx.doi.org/10.1016/j.chemosphere.2015.09.094
0045-6535/

being less active than expected based on the sum of the effects of the individual chemicals. The underlying mechanism of these less-than-additive effects can be, for instance, receptor blockage or inactivation; or enzyme induction (causing increased biotransformation). Chemicals may also enhance each other's effects, leading to a joint toxic effect being more-than-additive. Compared to antagonistic effects, relatively few clear examples of these obviously unwanted and so-called synergistic effects are available in the toxicological literature (Boobis et al., 2011; Cedergreen, 2014). Documented cases of obvious synergism include e.g. the mixture toxicity of piperonyl butoxide and pyrethroids in insects (Amweg et al., 2006) and atrazine and organophosphate insecticides in aquatic invertebrates (Pape-Lindstrom and Lydy, 1997; Belden and Lydy, 2000). Theoretically, synergism can be caused by e.g. metabolic enzyme induction or inhibition, leading to increased bioactivation or reduced biotransformation, respectively; chemical interactions (reactions leading to more toxic end products), increased availability/uptake (for instance by increased membrane permeability), or interactions between chemicals or with co-factors at the level of the receptor. If synergistic effects are biologically-relevant, i.e., occur in biological systems under environmentally-relevant conditions, the current risk assessment approach would be insufficient. Logically, synergism has therefore recently gained increased scientific, public, and regulatory interest (Backhaus et al., 2013; Cedergreen, 2014).

In previous work, we exposed recombinant yeast and mammalian cell lines to a series of crude oils and refined petroleum products, these being complex mixtures of thousands of individual compounds, and observed clear mixture effects (Vrabie et al., 2009, 2010, 2011). These mostly included antagonistic effects at the level of the A*h*, estrogen, and androgen receptor. Interestingly, one oil (a commercial engine oil for cars) was found to cause a clear dose-dependent synergistic effect in recombinant yeast containing the human androgen receptor (AR) when co-dosed with testosterone. Because knowledge of synergistic toxic effects and causative compounds is limited, in particular for the androgenic system, the present study was devoted to investigating the synergistic effect of this particular petroleum product in more detail. To this end, the oil was fractionated according to an open-column fractionation procedure, yielding so-called SARA fractions containing either 'saturates' (i.e. mainly aliphatic or alkylated compounds), 'aromatics', 'resins' (i.e. compounds regarded as more polar than hydrocarbons), or 'asphaltenes' (i.e. somewhat higher molecular weight compounds, again of unknown character). These fractions and combinations thereof were tested in the AR yeast assay. Additionally, we applied comprehensive two dimensional gas chromatography - mass spectrometry (GCxGC-MS), together with Fourier transform Infrared (FTIR) spectroscopy, ultraviolet–visible (UV-VIS) spectroscopy, high temperature GC-flame ionisation detection (FID), and nuclear magnetic resonance (NMR) spectroscopy, in an attempt to identify the causative synergistic compound(s). The combined use of fractionation, toxicity testing, and analysis by GCxGC-MS has previously proved to be extremely useful for identifying toxic components in oil (e.g. Booth et al., 2007; Booth et al., 2008; Rowland et al., 2011; Scarlett et al., 2011).

2. Material and methods

2.1. Chemicals

Solvents used were n-hexane (Pestiscan grade; Lab Scan, Dublin, Ireland), dichloromethane and methanol (HPLC grade; Lab Scan), ethanol (LiChrosolv grade; Merck, Darmstadt, Germany), and toluene (Spectranal grade; Riedel-de Haën, Seelze, Germany). Bacto-agar, dextrose, and yeast nitrogen base without amino acids and ammonium sulfate were purchased from Becton Dickinson (Breda, the Netherlands). Ammonium sulfate, L-leucine, 17β-estradiol, and testosterone were obtained from Sigma–Aldrich (Zwijndrecht, the Netherlands). Aluminum oxide (90 active neutral) and anhydrous sodium sulfate (analytical grade) were from Merck. A commercially-available, colourless, viscous lubricant (engine) oil for cars was bought at a local gas station in Wageningen, the Netherlands (Vrabie et al., 2009).

2.2. Yeast and culturing conditions

Recombinant yeast stably transfected with the human androgen receptor and the yeast enhanced green fluorescent protein as a reporter protein was purchased from the Institute of Food Safety (RIKILT), Wageningen, the Netherlands. A detailed description of the recombinant yeast can be found in (Bovee et al., 2007). Three days prior to running an assay, cultures were prepared by inoculating yeast on agar supplemented with L-leucine and incubating at 30 °C. After 48 h, one colony of yeast was added to 15 mL of minimal medium, containing 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 20 g/L dextrose, and 6 g/L L-leucine. The resulting suspensions were incubated for 24 h at 30 °C on an orbital shaker operating at 225 rpm. The cultures were then diluted in the above-mentioned medium to obtain an optical density of 0.04, as measured at 630 nm using a Shimadzu UV-160A spectrophotometer.

2.3. Oil fractionation

The oil was fractionated into so-called 'saturates', 'aromatics', 'resins', and 'asphaltenes' (SARA fractionation) as described in detail in the Supporting Information of Vrabie et al. (2012). In short, 100 mg of oil was first washed 10 times with *n*-hexane to precipitate out the asphaltenes. The asphaltenic residue was dried under nitrogen gas and dissolved in toluene. The pooled *n*-hexane phase was concentrated to 1 mL using a modified Kuderna-Danish apparatus and nitrogen, and separated by open column chromatography on neutral aluminum oxide into saturates, aromatics, and resins, by using *n*-hexane, dichloromethane, and methanol, respectively. In an attempt to recover any compounds left behind on the column, after the last elution step the column material was extracted with toluene, yielding a 'column extract' as an additional fraction. All fractions were finally exchanged to and diluted in ethanol. The oil concentrations in each of the resulting fractions obviously were unknown, but as a result of the dilutions applied, the total concentration in the pooled fractions corresponded to an exposure concentration (in the yeast assay) of 50 mg/L. This concentration was chosen, because when co-dosed with the EC_{50} of testosterone, it yielded a response of about 75% of the maximum testosterone response (Vrabie et al., 2010), which can be considered a clear and significant synergistic response.

2.4. Yeast exposure

For the experiments exposing yeast to pure oil or testosterone only (see Fig. 1), 200 μL of yeast suspension was pipetted into the inner 60 wells of a Greiner V bottom-shaped 96-well plate. Next, 2 μL of test solution containing either oil or testosterone in ethanol was added to each well. The outer wells received 200 μL of sterile water. In case of combination experiments (i.e., exposure to oil (fractions) plus testosterone), the yeast suspension

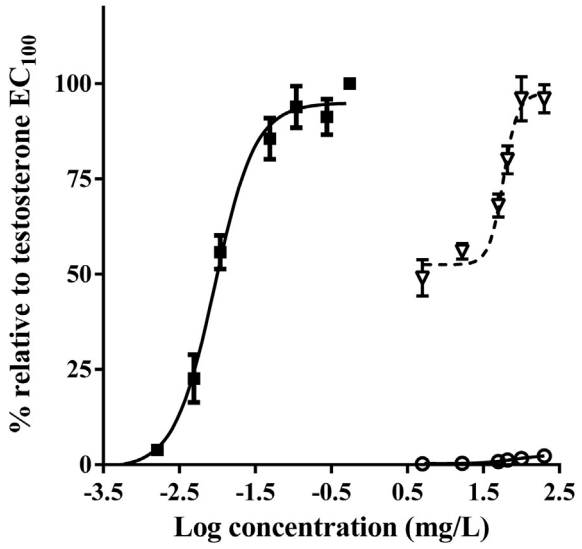

Fig. 1. Responses of recombinant yeast stably transfected with the human androgen receptor exposed to a concentration range of testosterone (0.002–0.6 mg/L; solid squares), a concentration range of engine oil (5–200 mg/L; open circles), and a combination of a fixed concentration of testosterone (EC_{50}; 0.01 mg/L) and a concentration range of engine oil (5–200 mg/L; open triangles). Error bars represent standard deviations (n = 3; intraplate variation of the experiment presented).

already contained 40 nM (0.01 mg/L) of testosterone, i.e., the compound's EC_{50}. In both experiments, the final ethanol concentration (<2%) was below cytotoxic thresholds and did not cause interference with later fluorescence measurements. Each plate included a full concentration range of testosterone (6–2000 nM, i.e., 0.002–0.6 mg/L; positive control), solvent controls (1 or 2% of ethanol), medium controls, and negative controls (0.6 nM of 17β-estradiol). Each sample, control, or standard concentration was tested in triplicate and the experiments were replicated three times. After dosing, the plates were incubated at 30 °C and 225 rpm for 24 h. Then, fluorescence was excited at 485 nm and measured at 530 nm on a Polar Star Galaxy fluorescence plate reader (BMG Labtech).

Results were expressed as percentage fluorescence formation relative to the maximal response induced by testosterone (EC_{100}) after subtracting the background (solvent control) response. Responses to the procedural (fractionation) blanks were all below 1% (results not shown) and were not adjusted for. Testosterone and oil data were analyzed with Prism GraphPad 4 software, using nonlinear regression according to a sigmoidal dose–response curve with variable slope. Data involving single concentration experiments (oil fractions with testosterone) were analyzed with GraphPad 4, using a two sample Student's *t*-test, assuming equal variances (α = 0.05).

2.5. Chemical analyses

GCxGC-MS analyses were conducted similarly to those reported by West et al. (2013). A full description is given in the supplementary information. Fourier Transform infrared (FT-IR) spectroscopy of the saturates fraction was performed with a Bruker Optics Alpha FT-IR spectrometer. Ultraviolet–visible (UV-VIS) spectra of solutions of the saturates fraction in dichloromethane were recorded on an Agilent/Hewlett Packard model 8453 spectrophotometer (Agilent Technologies, Waldbronn, Germany), using a wavelength range between 190 and 1100 nm, and a slit width of 1 nm. High temperature GC-flame ionisation detection (FID) was conducted as detailed by Sutton et al. (2010). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the saturates fraction were obtained in deuterated chloroform and the chemical shifts were measured relative to the solvent ($CDCl_3$; ^{1}H: 7.24 ppm; ^{13}C: 77.0 ppm), using a JEOL ECP-400 NMR spectrometer.

3. Results and discussion

3.1. Responses to unfractionated oil

Dose-response curves determined in the present study for AR yeast exposed to testosterone, the engine oil, and the combination of both, are presented in Fig. 1. The sigmoidal curve for testosterone demonstrates the validity of the assay (Bovee et al., 2007) and indicates the experiments were performed correctly (closed squares in Fig. 1). The absence of a clear dose–response curve for the oil tested alone (i.e., a response hardly surpassing the background value over the entire concentration range tested) therefore suggests that the petroleum product does not contain AR agonists (open circles in Fig. 1), at least not ones being active at the concentration range tested. Exposure of the yeast to the combination of a fixed concentration of testosterone (the EC_{50}) and a concentration range of the engine oil however resulted in a sigmoidal dose–response relationship (open triangles in Fig. 1). This observation clearly demonstrates the synergistic potency of the oil and confirms our previous results (Vrabie et al., 2010). Since the oil does not contain AR agonists, in case of additivity a more or less straight line around the 50% response level would have been observed for the entire oil concentration range investigated. To obtain more information on the chemical nature of the synergistic compound(s), the oil was fractionated.

3.2. Responses to separate SARA fractions

The oil fractionation yielded five fractions, which were dosed to the yeast at a single concentration, each in combination with the EC_{50} of testosterone. The results of the experiment are shown in Fig. 2. Remarkably, none of the exposures resulted in a response that came close to the response observed upon exposure to the combination of unfractionated oil plus testosterone (indicated by the dark grey bar), although exposure to the saturates fraction and testosterone gave a response that was significantly higher than the response to testosterone (EC_{50}) alone. In other words, the syner-

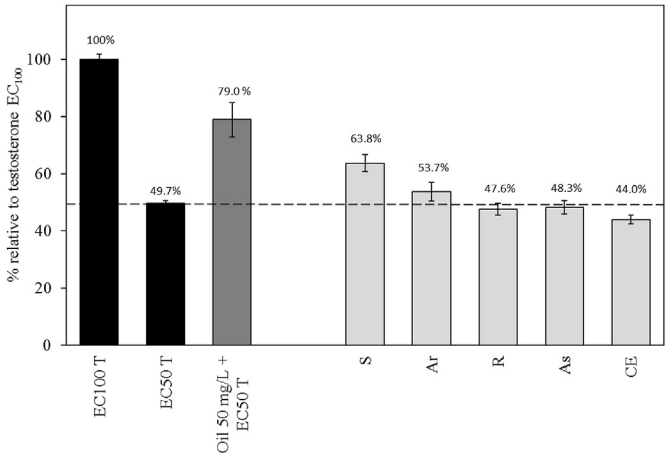

Fig. 2. Responses of recombinant yeast stably transfected with the human androgen receptor to the EC_{100} (0.6 mg/L) and EC_{50} (0.01 mg/L) of testosterone (black bars), a combination of the EC_{50} of testosterone and unfractionated engine oil (50 mg/L; dark grey bar), and a combination of the EC_{50} of testosterone and individual oil fractions obtained by SARA fractionation (light grey bars). Error bars represent standard deviations (n = 3; intraplate variation of the experiment presented). Explanation of abbreviations: T = testosterone; S = saturates fraction; Ar = aromatic fraction; R = resin fraction; As = asphaltenic fraction; CE = column extract.

gistic response as observed in Fig. 1 could not be fully reproduced after fractionation.

The reduction (saturates fraction) or loss of synergism may be explained in two ways. First, the causative compound(s) may be lost during the fractionation work, for instance by sorption to glassware or evaporation during concentration steps. Second, multiple compounds from different fractions may be needed for the overall synergistic response to occur. To investigate these possibilities, aliquots of the fractions were combined again to obtain a composite oil sample. This sample was combined with the EC_{50} of testosterone and dosed to the yeast. As shown in Fig. 3, (dark grey bar) this exposure caused a response that was close to the original result (see dark grey bar in Fig. 2), supporting the hypothesis that the synergistic response is caused by compounds present in different fractions. Although the response of the combined fractions (70.6 ± 4.7%) was somewhat (but not significantly) lower than the original one (79.0 ± 6.0%), the EC_{50} response of testosterone in the assay (Fig. 3) was also (significantly; t-test) lower at 44.8 ± 0.03%, compared to 49.7 ± 1.2% previously (Fig. 2). This lower response simply indicates a somewhat lower sensitivity of the first assay (within the natural variation), and this presumably compensates for the apparent loss in synergistic responses in the consecutive experiments. On the other hand, some losses of synergistic compounds during fractionation cannot fully be excluded. In order to investigate which fractions are involved in the synergism, combinations of two or three different separate fractions were prepared, which were subsequently combined with the testosterone EC_{50} and dosed to the yeast. Combining the fractions was done such that the exposure concentrations were the same as when dosing the separate fractions (i.e., by adapting the dilution factors).

3.3. Responses to combined SARA fractions

The results of the exposures to the pooled fractions are presented in Fig. 3. When combining the results of this figure and those of Fig. 2, it can be deduced that the synergistic compounds are not associated with the aromatic, asphaltene, or column extract fractions. Instead, as already discussed above, the saturates fraction seems to contain the compound(s) that contribute most to the overall synergistic response. The presence of the resin fraction (which neither is able to produce a synergistic response by itself, nor in combination with the aromatic fraction) is however required in order to significantly further increase the response and obtain the full synergistic level. The addition of the other fractions does not further (significantly) increase the response. In conclusion, compounds from the saturates and the resin fraction seem to be able to interact and jointly increase the response of the AR yeast to testosterone. This conclusion implies that at least three chemically distinct (classes of) compounds are involved in the response: the natural hormone and agonist of the receptor (testosterone), a compound (or compounds) from the saturates fraction, and a resin-type compound (or compounds). Because the fractions contain thousands of individual compounds, three compounds should be considered the minimum.

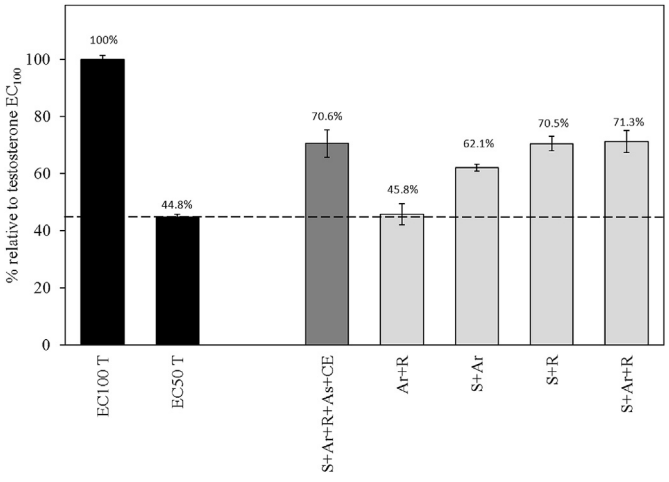

Fig. 3. Responses of recombinant yeast stably transfected with the human androgen receptor to the EC_{100} (0.6 mg/L) and EC_{50} (0.01 mg/L) of testosterone (black bars), composite oil (all fractions combined) plus the EC_{50} of testosterone (dark grey bar), and combinations of two or three SARA fractions plus the EC_{50} of testosterone (light grey bars). Error bars represent standard deviations (n = 3; intraplate variation of the experiment presented). Explanation of abbreviations: T = testosterone; S = saturates fraction; Ar = aromatic fraction; R = resin fraction; As = asphaltenic fraction; CE = column extract.

The conclusion that not two, but more compounds can jointly cause synergism is not unique. Synergism caused by multiple compounds for instance has been observed by Kunz and Fent (2006) in yeast stably expressing the human estrogen receptor. These authors however studied combinations of chemically similar compounds (UV filters), and to the best of our knowledge the present paper is the first demonstrating synergistic effects related to the androgenic system most probably caused by multiple compounds belonging to different chemical classes. Furthermore, the synergistic effects observed by Kunz and Fent amounted to about 10% of basal activity, whereas the present synergism (oil plus EC_{50} of testosterone) increased up to the full EC_{100} of testosterone (see Fig. 1). Also, because of the inclusion of the natural AR agonist, the present experiments might be considered relatively representative of the natural situation in the body.

Although the present individual saturates and resins fractions were not further fractionated preparatively (e.g. by HPLC in an effects-directed approach; Brack, 2003), the presumption that the saturates fraction contains chemically-distinct compounds from that of the resins fraction directly follows from the experimental fractionation procedure. The saturates and the resins fractions are eluted by solvents with very different polarities (*n*-hexane for the saturates and methanol for the resins) and these are separated by an aromatics fraction eluted with dichloromethane. It is therefore very unlikely that the causative compounds from the resins fraction are saturate-like compounds (methanol is a very poor solvent for many hydrocarbons). Similarly, it is unlikely that the causative compounds from the saturates fraction are polar resin-like compounds.

The ultimate question of the present study obviously relates to the exact chemical nature of the causative compounds in the saturates and resins fractions of the oil tested. Of the 11 crude oils and petroleum products tested previously, the commercial engine oil investigated here was the only oil causing synergism (Vrabie et al., 2011). Engine oils are typically produced from a base hydrocarbon feedstock, modified by the addition of synthetic additives to modify properties such as viscosity. The additives tend to be proprietary and known only to particular manufacturers, although the general classes of many additives have been described (e.g. Pourhossaini et al., 2005). An additive (or additives) specific to this particular product may be one of the potential candidates for causing the synergism. Since the saturates fraction produced the biggest synergistic effect (Figs. 2 and 3), we concentrated efforts on characterisation of this fraction.

Examination of the saturates fraction by FTIR spectroscopy indicated spectral characteristics typical of saturated hydrocarbons (Fig. S1), consistent with the elution in the saturates SARA fraction. However, UV-VIS spectrophotometry of a concentrated solution of the saturates fraction also revealed characteristics more typical of aromatic hydrocarbons, such as alkylbenzenes (Fig. S2). Such hydrocarbons have combined aromatic and 'alkyl' or non-aromatic (saturate-like) chromatographic properties, consistent with their

presence in this nominally saturated hydrocarbon SARA fraction. In order to examine this result in more detail, GCxGC-MS was applied. This technique is a powerful method for fractionation of compounds over a carbon number range of about C_{8-40} (or greater if high temperature GC columns are used; Dutriez et al., 2009). The analysis revealed the presence of about 1600 resolved compounds (see Supplementary Information), most of which were tentatively assigned as linear and branched alkanes (viz: 'saturates'), but which also included the alkylbenzenes suggested by UV-VIS spectrophotometry (Fig. S3), as well as bibenzyl (1,2-dihydrostilbene; diphenylethane). This last compound was however also present in the procedural blank at about the same concentration and thus is likely to have been introduced during the fractionation procedure. As such, it can be ruled out as a causative compound (it does not originate from the oil). Most, if not all, of the other compounds identified by FTIR, UV-VIS, and GCxGC-MS are common in oils, including the oils tested previously, which showed no synergistic activity. Hence, we eliminated these as the major causative agents as well.

To examine the possibility that compounds with molecular weights exceeding those detectable by GCxGC-MS were present, we additionally examined the saturates fraction by high temperature GC. No such compounds up to about C_{100}, were detected however (Fig. S4). This suggests that the causative synergistic compound(s) were not amenable to even high temperature GC methods. Some viscosity index modifiers used in lubricating oils, such as the oil described here, have a wide range of molecular weights and average molecular weights far exceeding those amenable to HTGC. Several are oligomers, polymers, or co-polymers of hydrocarbons, such as ethene, propene and isobutene (e.g. Mortier et al., 2009; Rudick, 2013), sometimes with further modifications. Since they are all highly alkylated, some lower molecular weight (but $> C_{100}$) proportion of these might reasonably be soluble in hexane and be expected to elute in a saturates SARA fraction. We therefore finally examined the saturates fraction by NMR spectroscopy. The resultant NMR (^{1}H and ^{13}C) spectra were typical of those of saturated hydrocarbons admixed with a small proportion of alkylaromatics, including the bibenzyl introduced from the procedures (Figs. S 5-7). No evidence of, for example, alkene copolymers or other viscosity modifiers (Mortier et al., 2009), was found, but this might be difficult to obtain due to the overwhelming number of saturated C, H atoms relative to other moieties, in typical modifiers (e.g. Mortier et al., 2009). Therefore, whilst nothing was revealed by NMR spectroscopy, which suggested that a commercial additive (or proportion of an additive mixture) eluted in the saturates fraction, this could not be entirely ruled out. In summary, despite the extensive chemical investigations, the compound(s) causing, or contributing to, the synergistic effect remain a mystery at this point. Additional analyses using liquid chromatography – mass spectrometry with a range of ionization techniques suitable for detection of hydrophobic compounds e.g. electrospray ionization, atmospheric pressure chemical ionization, and atmospheric pressure photoionization (Rowland et al., 2014) could be employed to search for compounds not amenable to GC.

The mechanism underlying the synergistic response is also unclear. Petrochemical compounds from the oil may for instance be bioactivated by enzymatic actions to become AR agonists, act at the level of the AR, making it more receptive to testosterone, stimulate protein kinases (if present) in yeast, or increase the availability of testosterone, e.g., by increasing its solubility or enhancing yeast cell wall permeability. Because yeast is devoid of other receptors normally present in mammalian cells and has low biotransformation capacity (Bovee et al., 2007), the first hypothetical explanation is not very likely. This however leaves multiple alternative explanations and the actual mechanism of the synergistic response remains unknown. It also remains uncertain whether or not the results are biologically-relevant; mammalian in vitro AR assays and/or in vivo experiments would be required to disclose any biological responses due to overstimulation of the AR by the engine oil. The latter experiments would also be required to test whether or not the petrochemical compounds are bioavailable in vivo, i.e., are taken up from the gut and are transported to the cellular receptors. Obviously, the present in vitro experiments did not provide any information on these pharmacological processes and accompanying realistic (internal) exposure scenarios, but strictly focused on toxicological aspects (receptor activation).

Anyhow, our results do illustrate the potentially highly complex nature of mixture effects. They underline the fact that risk assessment of mixtures is challenging and may be hard to generalize.

Acknowledgements

The chemical analysis part of this work was supported by the European Research Council through an Advanced Investigators Grant (No. 228149) awarded to S.J. Rowland for project OUTREACH. We are grateful to Michael Wilde (PU) for obtaining the NMR spectra and to Professor Simon Belt (PU) for helpful discussions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2015.09.094

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