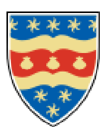

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Synergistic androgenic effect of a petroleum product caused by the joint action of at least three different types of compounds

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1

2

3 # Synergistic androgenic effects of a petroleum product caused by
4 # the joint action of at least three chemically distinct compounds

5

6

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9

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22

23

24 Key words: synergism, mixture toxicity, yeast, androgen receptor, petroleum, SARA.
25

26

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28 **Abstract** (200 words max)

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

45

46 ## 1. Introduction

47 Although humans and wildlife are exposed to complex mixtures of chemicals, current risk
48 assessment practice is based on individual chemicals. Generally, exposure concentrations of
49 single chemicals are compared to safe thresholds of the respective compounds, with the most
50 sophisticated approach being the application of concentration addition or independent action
51 concepts, i.e., the assumption that effects of separate chemicals can be added up (Brackhaus et
52 al., 2013). It is well-known however, that mixture toxicity caused by interactive effects of
53 multiple chemicals may potentially occur. For example, numerous cases of antagonism exist
54 in the toxicological literature, describing a joint effect of chemicals being less active than
55 expected based on the sum of the effects of the individual chemicals. The underlying
56 mechanism of these less-than-additive effects can be, for instance, receptor blockage or
57 inactivation, or enzyme induction (causing increased biotransformation). Antagonism does
58 not complicate risk assessment *per se*, as it will result in a conservative (overprotective)
59 assessment.

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60 Chemicals may however also enhance each other's effects, leading to a joint toxic effect being
61 more-than-additive. Compared to antagonistic effects, relatively few clear examples of these
62 obviously unwanted and so-called synergistic effects, are available in the toxicological
63 literature (Boobis et al., 2011; Cedergreen, 2014). Documented cases of obvious synergism
64 include e.g. the mixture toxicity of piperonyl butoxide and pyrethroids in insects (Amweg et
65 al., 2006) and atrazine and organophosphate insecticides in aquatic invertebrates (Belden and
66 Lydy, 2000). Theoretically, synergism can be caused by e.g. metabolic enzyme induction or
67 inhibition, leading to increased bioactivation or reduced biotransformation, respectively;
68 chemical interactions (reactions leading to more toxic end products), increased
69 availability/uptake (for instance by increased membrane permeability), or interactions
70 between chemicals or with co-factors at the level of the receptor. If synergistic effects are
71 biologically-relevant, i.e., occur in biological systems under environmentally-relevant
72 conditions, the current risk assessment approach would be insufficient. Logically, synergism
73 has therefore recently gained increased scientific, public, and regulatory interest (Brackhaus et
74 al., 2013; Cedergreen, 2014).
75 In previous work, we exposed recombinant yeast and mammalian cell lines to a series of
76 crude oils and refined petroleum products, these being complex mixtures of thousands of
77 individual compounds, and observed clear mixture effects (Vrabie et al., 2009, 2010, 2011).
78 These mostly included antagonistic effects at the level of the A*h*, estrogen, and androgen
79 receptor. Interestingly, one oil (a commercial engine oil for cars) was found to cause a clear
80 dose-dependent synergistic effect in recombinant yeast containing the human androgen
81 receptor (AR) when co-dosed with testosterone. Because knowledge on synergistic toxic
82 effects and causative compounds is limited, in particular for the androgenic system, the
83 present study was devoted to investigating the synergistic effect of this particular petroleum
84 product in more detail. To this end, the oil was fractionated according to an open-column
85 fractionation procedure, yielding so-called SARA fractions containing either: 'saturates' (i.e.
86 mainly aliphatic or alkylated compounds), 'aromatics', 'resins' (i.e. compounds regarded as
87 more polar than hydrocarbons), or 'asphaltenes' (i.e. somewhat higher molecular weight
88 compounds, again of unknown character). These fractions and combinations thereof were
89 tested in the AR yeast assay. Additionally, we applied comprehensive two dimensional gas
90 chromatography - mass spectrometry (GCxGC-MS), together with Fourier transform Infrared
91 (FTIR) spectroscopy and ultraviolet-visible (UV-VIS) spectroscopy in an attempt to identify
92 the causative synergistic compound(s). The combined use of fractionation, toxicity testing,

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93 and analysis by GCxGC-MS has previously proved to be extremely useful for identifying
94 toxic components in oil (e.g. Booth et al., 2007; Booth et al., 2008; Rowland et al., 2011;
95 Scarlett et al., 2011).
96

97 2. Material and Methods

98 2.1. Chemicals

99 Solvents used were n-hexane (Pestiscan grade; Lab Scan, Dublin, Ireland), dichloromethane
100 and methanol (HPLC grade; Lab Scan), ethanol (LiChrosolv grade; Merck, Darmstadt,
101 Germany), and toluene (Spectranal grade; Riedel-de Haën, Seelze, Germany). Bacto-agar,
102 dextrose, and yeast nitrogen base without amino acids and ammonium sulfate were purchased
103 from Becton Dickinson (Breda, the Netherlands). Ammonium sulfate, L-leucine, 17β-
104 estradiol, and testosterone were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).
105 Aluminum oxide (90 active neutral) and anhydrous sodium sulfate (analytical grade) were
106 from Merck. A commercially-available, colorless, viscous lubricant (engine) oil for cars
107 (Visco 2000 Diesel; 15W-40) was bought at a local BP gas station in Wageningen, the
108 Netherlands.
109

110 2.2. Yeast and culturing conditions

111 Recombinant yeast stably transfected with the human androgen receptor and the yeast
112 enhanced green fluorescent protein as a reporter protein was purchased from the Institute of
113 Food Safety (RIKILT), Wageningen, the Netherlands. Three days prior to running an assay,
114 cultures were prepared by inoculating yeast on agar supplemented with L-leucine and
115 incubating at 30 ºC. After 48 h, one colony of yeast was added to 15 mL of minimal medium,
116 containing 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 20 g/L dextrose, and 6 g/L
117 L-leucine. The resulting suspensions were incubated for 24 h at 30 ºC on an orbital shaker
118 operating at 225 rpm. Then, the cultures were diluted in the above-mentioned medium to
119 obtain an optical density of 0.04, as measured at 630 nm using a Shimadzu UV-160A
120 spectrophotometer.
121

122 2.3. Oil fractionation

123 The oil was fractionated into so-called saturates, aromatics, resins, and asphaltenes (SARA
124 fractionation) as described in detail in the supporting information of Vrabie et al. (2012). In
125 short, 100 mg of oil was first washed 10 times with *n*-hexane to precipitate out the

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126 asphaltenes. The asphaltenic residue was dried under nitrogen gas and dissolved in toluene.
127 The pooled *n*-hexane phase was concentrated to 1 mL using a modified Kuderna-Danish
128 apparatus and nitrogen, and separated by open column chromatography on neutral aluminum
129 oxide, using *n*-hexane, dichloromethane, and methanol as eluents, respectively. In order to try
130 not to exclude any compounds left behind on the column, after the last elution step the column
131 material was extracted with toluene, yielding a 'column extract' as an additional fraction. All
132 fractions were finally exchanged to and diluted in ethanol. The oil concentrations in each of
133 the resulting fractions obviously were unknown, but as a result of the dilutions applied, the
134 total concentration in the pooled fractions corresponded to an exposure concentration (in the
135 yeast assay) of 50 mg/L. This concentration was chosen, because when co-dosed with the
136 EC_{50} of testosterone, it yielded a response of about 75% of the maximum testosterone
137 response (Vrabie et al., 2010), which can be considered a clear and significant synergistic
138 response.
139
140 **2.4. Yeast exposure**
141 In the experiments during which yeast was exposed to the pure oil or testosterone only, 200
142 μL of yeast suspension was pipetted into the inner 60 wells of a Greiner V bottom-shaped 96-
143 well plate. Next, 2 μL of test solution containing either oil or testosterone in ethanol was
144 added to each well. The outer wells received 200 μL of sterile water. In case of combination
145 experiments (i.e., exposure to oil (fractions) plus testosterone), the yeast suspension already
146 contained 40 nM (0.01 mg/L) of testosterone, i.e., the compound's EC_{50}. In both experiments,
147 the final ethanol concentration (< 2%) was below cytotoxic thresholds and did not cause
148 interference with later fluorescence measurements. Each plate included a full concentration
149 range of testosterone (6-2000 nM; positive control standard), solvent controls (1 or 2 % of
150 ethanol), medium controls, and negative controls (0.6 nM of 17β-estradiol). Each sample,
151 control, or standard concentration was tested in triplicate and the experiments were replicated
152 three times. After dosing, the plates were incubated at 30 ºC and 225 rpm for 24 hours. Then,
153 fluorescence was measured at an excitation and emission wavelength of 485 and 530 nm,
154 respectively, on a Polar Star Galaxy fluorescence plate reader (BMG Labtech).
155 Results were expressed as percentage fluorescence formation relative to the maximal response
156 induced by testosterone (EC_{100}), after subtracting the background (solvent control) response.
157 Testosterone and oil data were analyzed with Prism GraphPad 4 software, using nonlinear
158 regression according to a sigmoidal dose-response curve with variable slope. Data involving

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159 single concentration experiments (oil fractions with testosterone) were analyzed with
160 GraphPad 4, using a two sample Student's *t*-test, assuming equal variances (α=0.05).
161
162
163 **2.5. GCxGC-MS analyses**
164 GCxGC-MS analyses were conducted similarly to those reported by West et al (2013). A full
165 description is given in the supplementary information.
166
167 **2.6 Fourier Transform infrared (FTIR) spectroscopy, ultraviolet-visible absorption**
168 **spectrophotometry (UV-VIS), high temperature GC-flame ionisation detection (FID)**
169 **and nuclear magnetic resonance (NMR) spectroscopy**

170 FTIR spectroscopy of the 'saturates' fraction was performed with a Bruker Optics Alpha FT-
171 IR spectrometer. UV spectra of solutions of the 'saturates' fraction in dichloromethane were
172 recorded on an Agilent/Hewlett Packard model 8453 (Agilent Technologies, Waldbronn,
173 Germany), using a wavelength range between 190 and 1100 nm, and a slit width of 1 nm.
174 High temperature GC-FID was conducted as detailed by Sutton et al. (2010). ^{1}H and ^{13}C
175 NMR spectra of the 'saturates' fraction were obtained in deuterated chloroform and the
176 chemical shifts were measured relative to the solvent ($CDCl_3$; ^{1}H: 7.24 ppm; ^{13}C: 77.0 ppm),
177 using a JEOL ECP-400 NMR spectrometer.

178
179
180 **3. Results and Discussion**
181 **3.1. Responses to unfractionated oil**
182 In Figure 1, dose-response curves of AR yeast exposed to testosterone, the engine oil, and the
183 combination of both are presented. The sigmoidal curve for testosterone demonstrates the
184 validity of the assay (Bovee et al., 2007) and indicates the experiments were performed
185 correctly. The absence of a clear dose-response curve for the oil tested alone (i.e., a response

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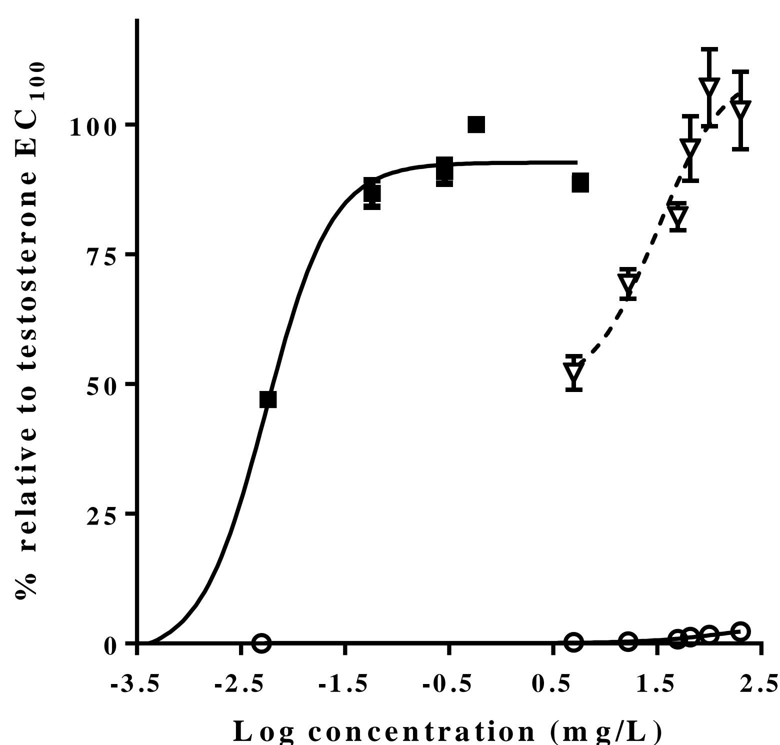
186 hardly surpassing the background value over the entire concentration range tested) therefore
187 shows that the petroleum product does not contain AR agonists. Exposure of the yeast to the
188 combination of a fixed concentration of testosterone (its EC_{50}) and a concentration range of
189 the engine oil however resulted in a sigmoidal dose-response relationship (open triangles in
190 Figure 1). This observation clearly demonstrates the synergistic potency of the oil (Vrabie et
191 al., 2010). Since the oil does not contain AR agonists, in case of additivity a more or less
192 straight line around the 50% response level would have been observed for the entire oil
193 concentration range investigated. To obtain more information on the chemical nature of the
194 synergistic compound(s), the oil was fractionated.
195



196
197 ***Figure 1***. *Responses of recombinant yeast stably transfected with the human androgen*
198 *receptor exposed to a concentration range of testosterone (0.002-0.6 mg/L; solid squares), a*
199 *concentration range of engine oil (5-200 mg/L; open circles), and a combination of a fixed*
200 *concentration of testosterone (EC_{50}; 0.01 mg/L) and a concentration range of engine oil (5-*
201 *200 mg/L; open triangles).*
202
203 **3.2. Responses to separate SARA fractions**

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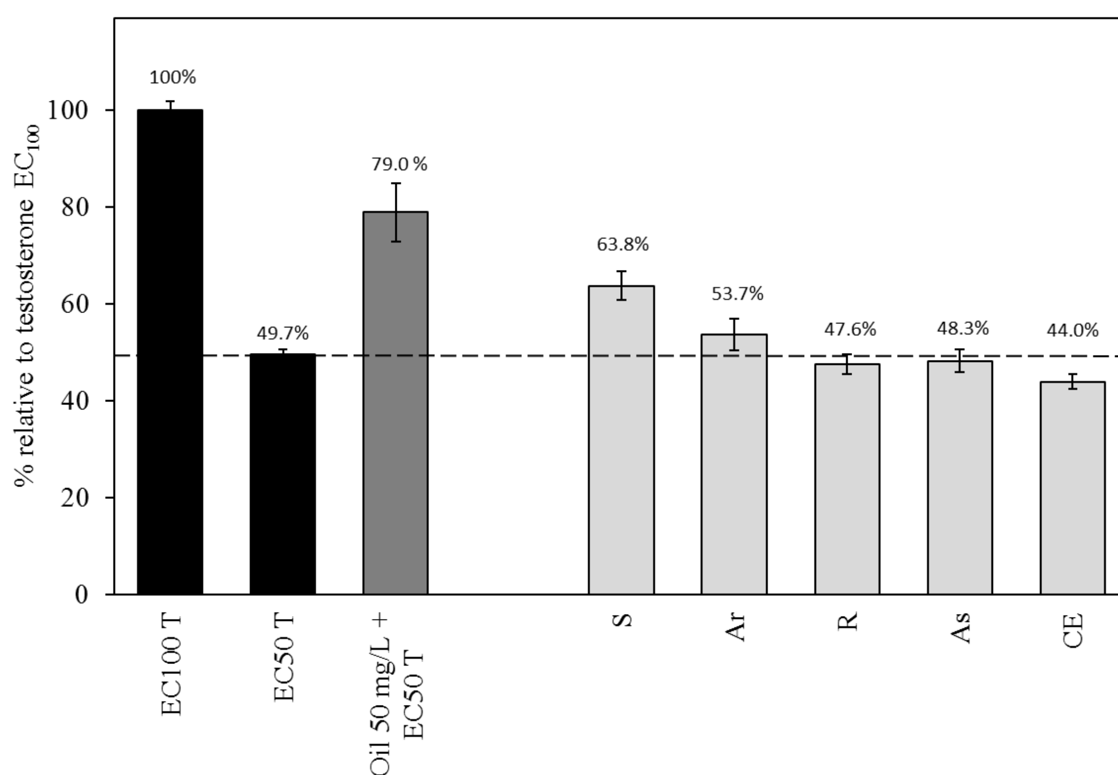
204 The oil fractionation yielded five fractions, which were dosed to the yeast at a single
205 concentration, each in combination with the EC_{50} of testosterone. The results of the
206 experiment are shown in Figure 2. Remarkably, none of the exposures resulted in a response
207 that came close to the response observed upon exposure to the combination of unfractionated
208 oil plus testosterone (indicated by the dark grey bar), although exposure to the saturates
209 fraction and testosterone gave a response that was significantly higher than the response to
210 testosterone (EC_{50}) alone. In other words, the synergistic response as observed in Figure 1
211 could not be fully reproduced after fractionation.
212



213
214
215 ***Figure 2***. *Responses of recombinant yeast stably transfected with the human androgen*
216 *receptor to the* EC_{100} *(0.6 mg/L) and* EC_{50} *(0.01 mg/L) of testosterone (black bars), a*
217 *combination of the* EC_{50} *of testosterone and unfractionated engine oil (50 mg/L; dark grey*
218 *bar), and a combination of the* EC_{50} *of testosterone and individual oil fractions obtained by*
219 *SARA fractionation (light grey bars). Explanation of abbreviations: T = testosterone; S =*
220 *saturates fraction; Ar = aromatic fraction; R = resin fraction; As = asphaltenic fraction; CE*
221 *= column extract.*

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222

223 The reduction ('saturates') or loss of synergism may be explained in two ways. First, the
224 causative compound(s) may be lost during the fractionation work, for instance by sorption to
225 glassware or evaporation during concentration steps. Second, multiple compounds from
226 different fractions may be needed for the overall synergistic response to occur. To investigate
227 these possibilities, aliquots of the fractions were combined again to obtain a composite oil
228 sample. This sample was combined with the EC_{50} of testosterone and dosed to the yeast. As
229 shown in Figure 3, (dark grey bar) this exposure caused a response that was close to the
230 original result (see dark grey bar in Figure 2), supporting the hypothesis that the synergistic
231 response is caused by compounds present in different fractions. Although the response of the
232 combined fractions (70.6 ± 4.7 %) was somewhat lower than the original one (79.0 ± 6.0 %) ,
233 the EC_{50} response of testosterone in the assay (Figure 3) was also lower at 44.8 ± 1.0 %,
234 compared to 49.7 ± 1.2 % previously (Figure 2). This lower response simply indicates a
235 lowered sensitivity of the assay, and this presumably compensates for the apparent loss in
236 synergistic responses in the consecutive experiments. On the other hand, some losses of
237 synergistic compounds during fractionation cannot fully be excluded. In order to investigate
238 which fractions are involved in the synergism, combinations of two or three different separate
239 fractions were prepared, which were subsequently combined with the testosterone EC_{50} and
240 dosed to the yeast. Combining the fractions was done such that the exposure concentrations
241 were the same as when dosing the separate fractions (i.e., by tuning the dilution factors).

242

243 **3.3. Responses to combined SARA fractions**

244 The results of the exposures to the pooled fractions are presented in Figure 3. When
245 combining the results of this Figure and those of Figure 2, it can be deduced that the
246 synergistic compounds are not associated with the aromatic, asphaltene, or column extract
247 fractions. Instead, as already discussed above, the saturates fraction seems to contain the
248 compound(s) that contribute most to the overall synergistic response. The presence of the
249 resin fraction (which neither is able to produce a synergistic response by itself, nor in
250 combination with the aromatic fraction) is however required in order to obtain the full
251 synergistic response. The addition of the other fractions does not further increase the
252 response. In conclusion, compounds from the saturates and the resin fraction seem to be able
253 to interact and jointly increase the response of the AR yeast to testosterone. This conclusion
254 implies that at least three chemically distinct (classes of) compounds are involved in the

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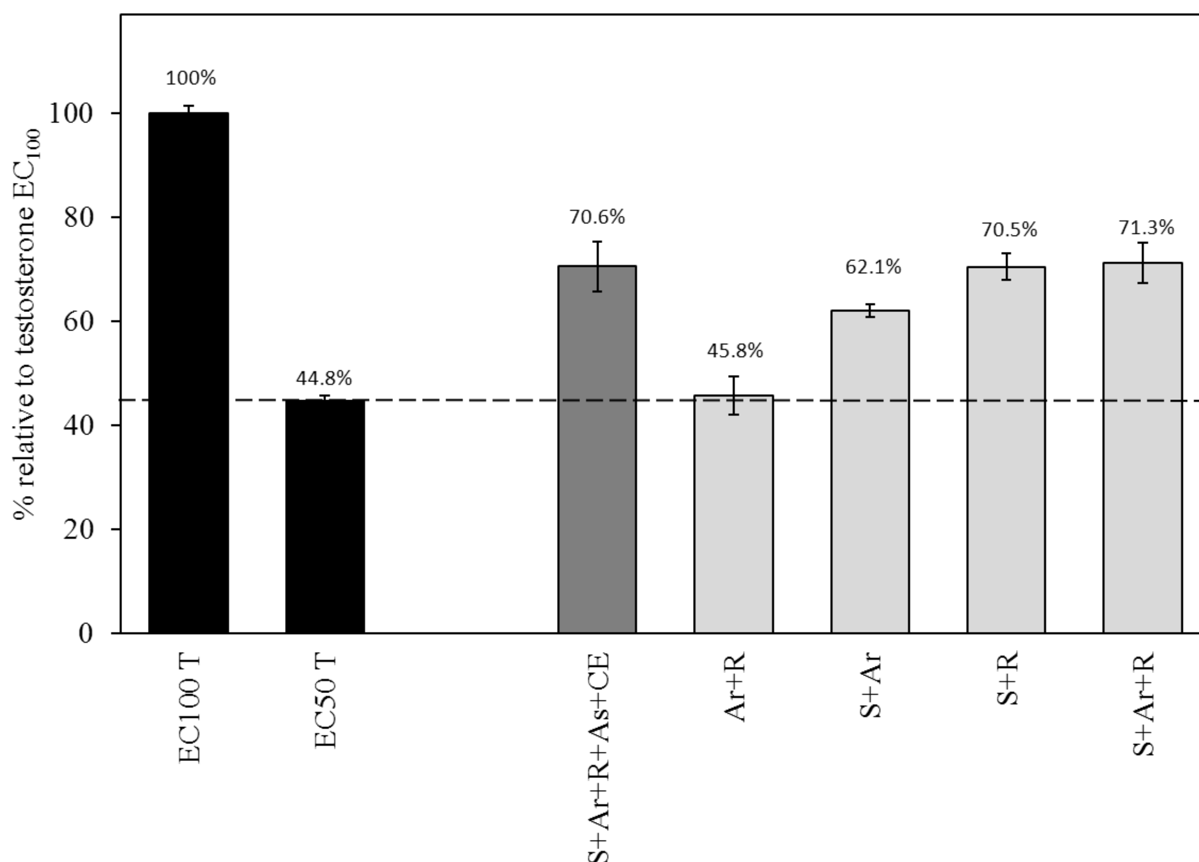
255 response: the natural hormone and agonist of the receptor (testosterone), a compound(s) from
256 the 'saturates' fraction, and a 'resin' compound(s). Because the fractions contain thousands of
257 individual compounds, three compounds should be considered the minimum.



258
259
260 ***Figure 3***. *Responses of recombinant yeast stably transfected with the human androgen*
261 *receptor to the* EC_{100} *(0.6 mg/L) and* EC_{50} *(0.01 mg/L) of testosterone (black bars), composite*
262 *oil (all fractions combined) plus the* EC_{50} *of testosterone (dark grey bar), and combinations of*
263 *two or three SARA fractions plus the* EC_{50} *of testosterone (light grey bars). Explanation of*
264 *abbreviations: T = testosterone; S = saturates fraction; Ar = aromatic fraction; R = resin*
265 *fraction; As = asphaltenic fraction; CE = column extract.*
266
267 The conclusion that not two, but more compounds can jointly cause synergism is not unique.
268 Synergism caused by multiple compounds for instance has been observed by Kunz and Fent
269 (2006) in yeast stably expressing the human estrogen receptor. These authors however studied
270 combinations of chemically similar compounds (UV filters), and to the best of our knowledge
271 the present paper is the first demonstrating synergistic effects related to the androgenic system

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272 caused by multiple compounds belonging to different chemical classes. Furthermore, the
273 synergistic effects observed by Kunz and Fent amounted to about 10% of basal activity,
274 whereas the present synergism (oil plus EC_{50} of testosterone) increased up to the full EC_{100} of
275 testosterone. Also, because of the inclusion of the natural AR agonist, the present experiments
276 might be considered relatively representative of the natural situation in the body.
277 Although the present individual 'saturates' and 'resins' fractions were not further fractionated
278 preparatively (e.g. by HPLC in an effects-directed approach; Brack, 2003), the presumption
279 that the 'saturates' fraction contains chemically-distinct compounds from that of the 'resins'
280 fraction, directly follows from the experimental fractionation procedure. The 'saturates' and
281 the 'resins' fractions are eluted by solvents with very different polarities (*n*-hexane for the
282 'saturates' and methanol for the 'resins') and these are separated by an 'aromatics' fraction
283 eluted with dichloromethane. It is therefore very unlikely that the causative compounds from
284 the 'resins' fraction are 'saturate'-like compounds (methanol is a very poor solvent for many
285 hydrocarbons). Similarly, it is unlikely that the causative compounds from the 'saturates'
286 fraction are polar 'resin'-like compounds.
287 The ultimate question of the present study obviously relates to the exact chemical nature of
288 the causative compounds in the 'saturates' and 'resins' fractions of the oil tested. Of the 11
289 crude oils and petroleum products tested previously, the commercial engine oil investigated
290 here was the only oil causing synergism (Vrabie et al., 2011). Engine oils are typically
291 produced from a base hydrocarbon feedstock, modified by the addition of synthetic
292 additives to modify properties such as viscosity. The additives tend to be proprietary and
293 known only to particular manufacturers, although the general classes of many additives have
294 been described (e.g. Pourhossaini et al., 2005). An additive(s) specific to this particular
295 product may be one of the potential candidates.
296
297 Since the 'saturates' fraction produced the biggest synergistic effect (Figures 2 and 3), we
298 concentrated efforts on characterisation of this fraction.
299
300 Examination by FTIR spectroscopy indicated spectral characteristics typical of saturated
301 hydrocarbons (Figure S1), consistent with the elution in the 'saturates' SARA fraction.
302
303 However, UV-VIS spectrophotometry of a concentrated solution of the 'saturates' fraction
304 also revealed characteristics more typical of aromatic hydrocarbons, such as alkylbenzenes

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305 (Figure S2). Such hydrocarbons have combined aromatic and 'alkyl' or non-aromatic
306 ('saturate'-like) chromatographic properties, consistent with their presence in this nominally
307 saturated hydrocarbon SARA fraction.
308 In order examine this in result more detail, multidimensional comprehensive gas
309 chromatography-mass spectrometry (GCxGC-MS) was applied. This technique is a powerful
310 method for fractionation of compounds over a carbon number range of about C_{8-40} (or greater
311 if high temperature GC columns are used; Dutriez et al., 2009). The analysis revealed the
312 presence of about 1600 resolved compounds, most of which were tentatively assigned as
313 linear and branched alkanes (viz: 'saturates'), but which also included the alkylbenzenes
314 suggested by UV-VIS spectrophotometry (Figure S3) as well as bibenzyl (1,2-
315 dihydrostilbene; diphenylethane), which was however, also in the procedural blank at about
316 the same concentration.
317
318 Since most, if not all, of the compounds identified by FTIR, UV-VIS and GCxGC-MS are
319 common in oils, including those oils tested previously which showed no androgenic activity,
320 we eliminated these as the major causative agents.
321
322 To examine the possibility that compounds with molecular weights exceeding those
323 detectable by GCxGC-MS were present, we also examined the 'saturates' fraction by high
324 temperature GC: no such compounds up to about C_{100}, were detected (Figure S4). This
325 suggests that the causative synergistic compound(s) were not amenable to even high
326 temperature GC methods.
327

328 Some viscosity index modifiers used in lubricating oils, such as the oil described here, have a
329 wide range of molecular weights and average molecular weights far exceeding those
330 amenable even to HTGC and several are oligomers, polymers, or co-polymers of
331 hydrocarbons, such as ethene, propene and isobutene (e.g. Mortier et al., 2009; Rudnick,
332 2013), sometimes with further modifications. Since they are all highly alkylated, some lower
333 molecular weight (but $>C_{100}$) proportion of these might reasonably be soluble in hexane and
334 be expected to elute in a 'saturates' SARA fraction. We therefore examined the 'saturates'
335 fraction by NMR spectroscopy.

336

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337 The resultant NMR (^{1}H and ^{13}C) spectra were typical of those of saturated hydrocarbons
338 admixed with a small proportion of alkylaromatics, including the bibenzyl introduced from
339 the procedures (Figures S 5-7). Resonances attributed to protons from saturates were present
340 in the proton spectrum at 0.5-1.5 ppm. Those attributed to aromatic protons were present at
341 7.1-7.3 ppm (Figure S5). (Resonances attributed to protons from the bibenzyl impurity were
342 observed at ca. 2.4 ppm in the proton spectrum (-C$\underline{H}_2$Ar) and 7.1-7.3 ppm (aromatic ring
343 protons) and those from a trace of dichloromethane solvent at ~5.3 ppm). Confirmatory
344 resonances were present in the ^{13}C spectra (e.g. carbons associated with alkyl (saturated)
345 chains 0-40 ppm; *ipso* aromatic ring carbon, ca. 137 ppm, absent from DEPT spectrum, other
346 aromatic ring carbons ca. 125-130 ppm; Figure S6). No evidence of, for example, alkene co-
347 polymers or other viscosity modifiers (Mortier et al., 2009), was found, but this might be
348 difficult to obtain due to the overwhelming number of saturated C,H atoms relative to other
349 moieties, in typical modifiers (e.g. Mortier et al., 2009). Therefore, whilst nothing was
350 revealed by NMR spectroscopy which suggested that a commercial additive (or proportion of
351 an additive mixture) eluted in the 'saturates' fraction, this could not be entirely ruled out. The
352 compound(s) causing or contributing to, the biological effects remain a mystery at this point.
353
354 The mechanism underlying the synergistic response is also unclear. Petrochemical compounds
355 from the oil may for instance be bioactivated by enzymatic actions to become AR agonists,
356 act at the level of the AR, making it more receptive to testosterone, stimulate protein kinases
357 (if present) in yeast, or increase the availability of testosterone, e.g., by increasing its
358 solubility or enhancing yeast cell wall permeability. Because yeast is devoid of other
359 receptors normally present in mammalian cells and has low biotransformation capacity
360 (Bovee et al., 2007), the first hypothetical explanation is not very likely. This however leaves
361 multiple alternative explanations and the actual mechanism of the synergistic response
362 remains unknown. It also remains uncertain whether or not the results are biologically-
363 relevant; mammalian in vitro AR assays and/or in vivo experiments would be required to
364 disclose any biological responses due to overstimulation of the AR by the engine oil.
365 Anyhow, the present results illustrate the highly complex nature of mixture effects. They
366 underline the fact that risk assessment of mixtures is challenging and may be hard to
367 generalize.
368
369

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370 **Acknowledgements**

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373 OUTREACH. We are grateful to Michael Wilde (PU) for obtaining the NMR spectra and to
374 Professor Simon Belt (PU) for helpful discussions.

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471 # Supplementary information

472

473 *GCxGC-MS*

474 GCxGC-MS analyses were conducted using an Agilent 7890A gas chromatograph (Agilent
475 Technologies, Wilmington, DE) fitted with a Zoex ZX2 GCxGC cryogenic modulator
476 (Houston, TX, USA) interfaced with an Almsco BenchTOFdx™ time-of-flight mass
477 spectrometer (Almsco International, Llantrisant, Wales, UK). The first-dimension column was
478 a 100% dimethyl polysiloxane 60 m x 0.25 mm x 0.25 µm Rxi®-1ms (Restek, Bellefonte,
479 USA), and the second-dimension column was a 50% phenyl polysilphenylene siloxane 2.5 m
480 x 0.1 mm x 0.1 µm BPX50 (SGE, Melbourne, Australia). Helium was used as carrier gas and
481 the flow was kept constant at 1.0 mL min^{-1}. Samples (1µL) were injected at 300°C splitless.
482 The oven was programmed from 40°C (hold for 1 min), then heated to 130°C at 10°C min^{-1}
483 then at 2°C min^{-1} to 320°C (held for 15 min). The modulation period was 6s. The MS transfer
484 line temperature was 290°C and ion source 300°C. Data processing was conducted using GC
485 Image™ v2.1 (Zoex, Houston, TX, USA).

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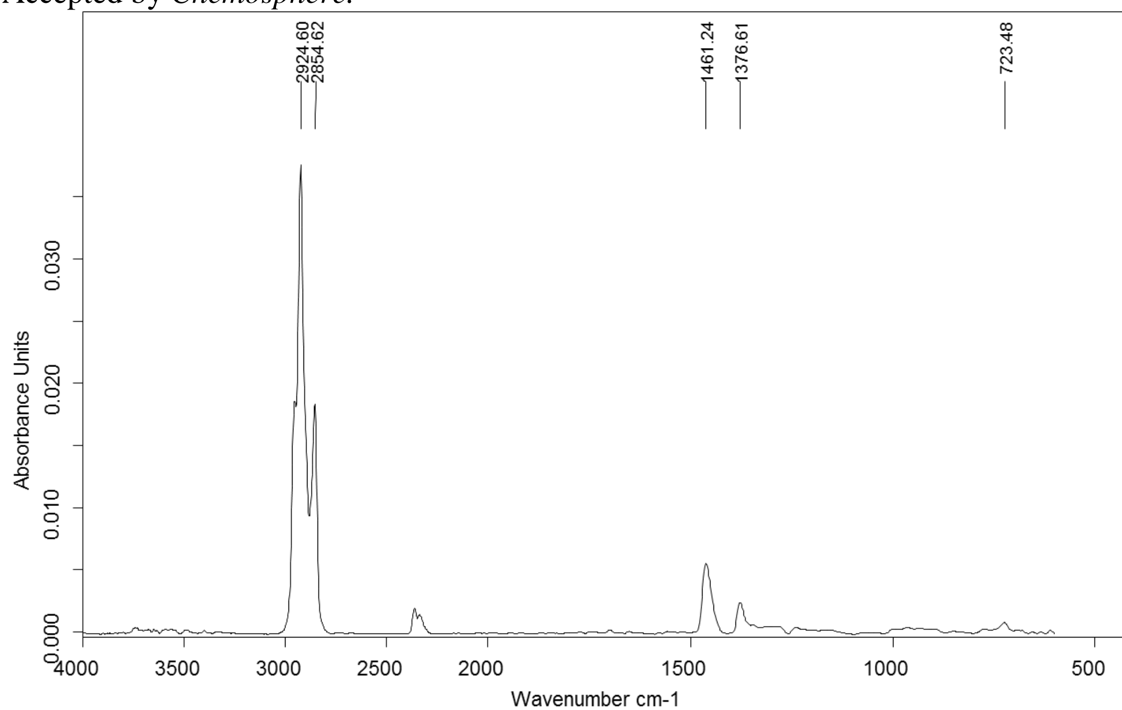

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491 **Figure S1**. Infrared spectrum of the 'saturates' fraction, showing typical absorbances
492 attributed to $-CH_2$, $-CH_3$ stretch (ca 2900 cm^{-1}) and bend (ca 1450 cm^{-1}) vibrations in
493 saturated hydrocarbon moieties.
494

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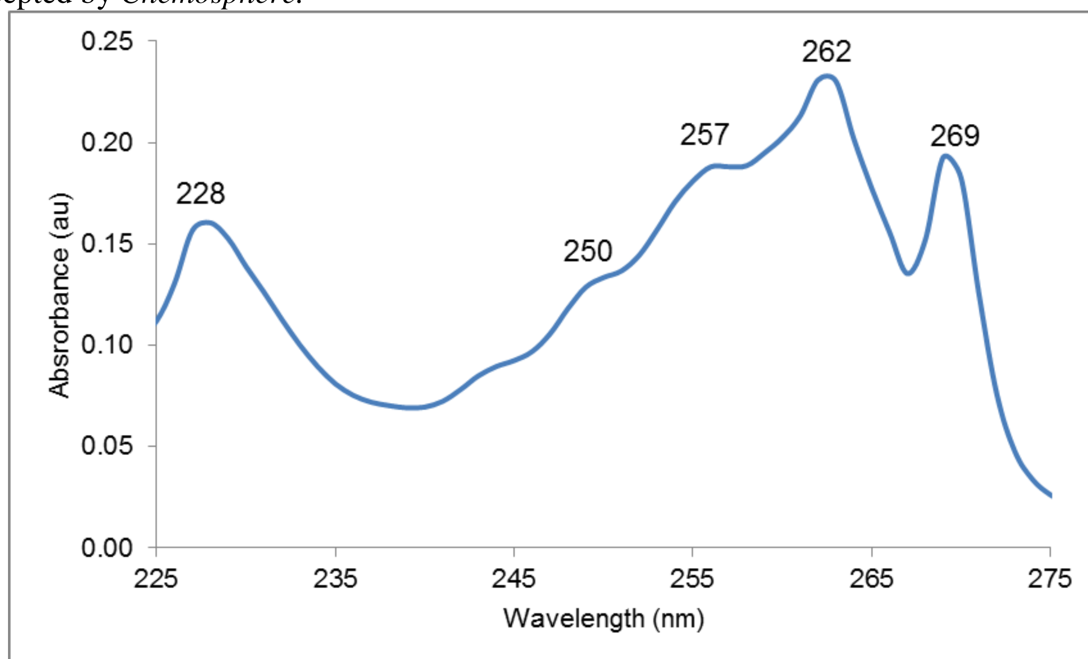

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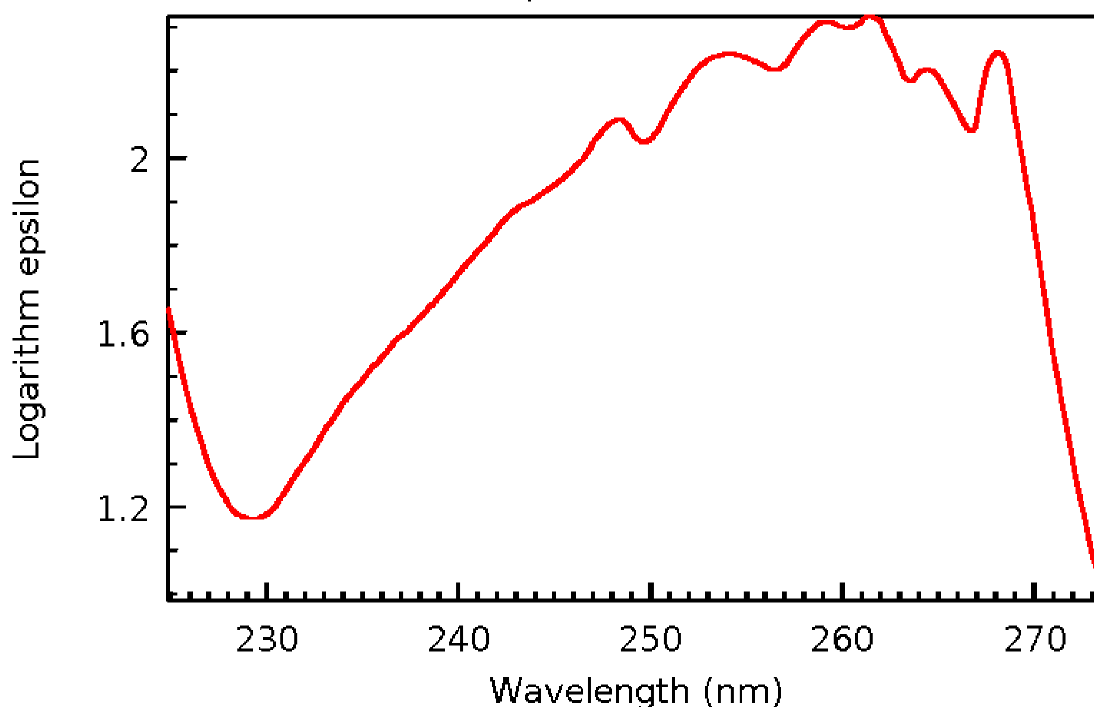

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499 **Figure S2**. Ultraviolet-visible absorption spectrum of the 'saturates' fraction (top) dissolved
500 in dichloromethane, showing absorbances similar to that those of butylbenzene (bottom), as
501 reported by NIST. The absorbance at 228 nm in the 'saturates' fraction is likely due to the
502 dichloromethane solvent.
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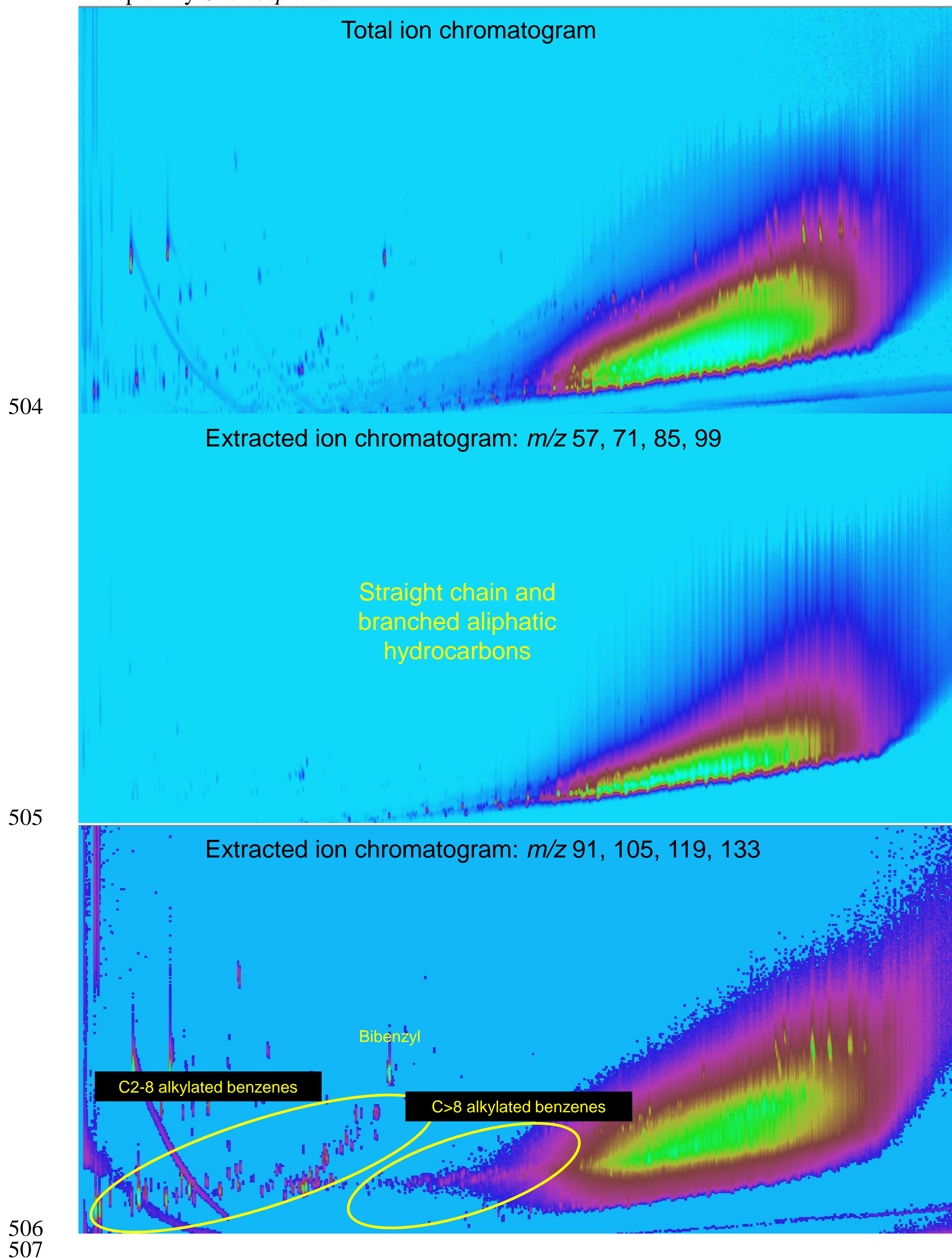

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508 **Figure S3**. GCxGC-MS chromatograms of the saturates fraction: (Top) total ion
509 chromatogram (top); (Middle) extracted ion chromatogram (EIC) m/z 57+71+85+99,
510 highlighting saturated hydrocarbons (Middle); and (Bottom) EIC m/z 91+105+119+133

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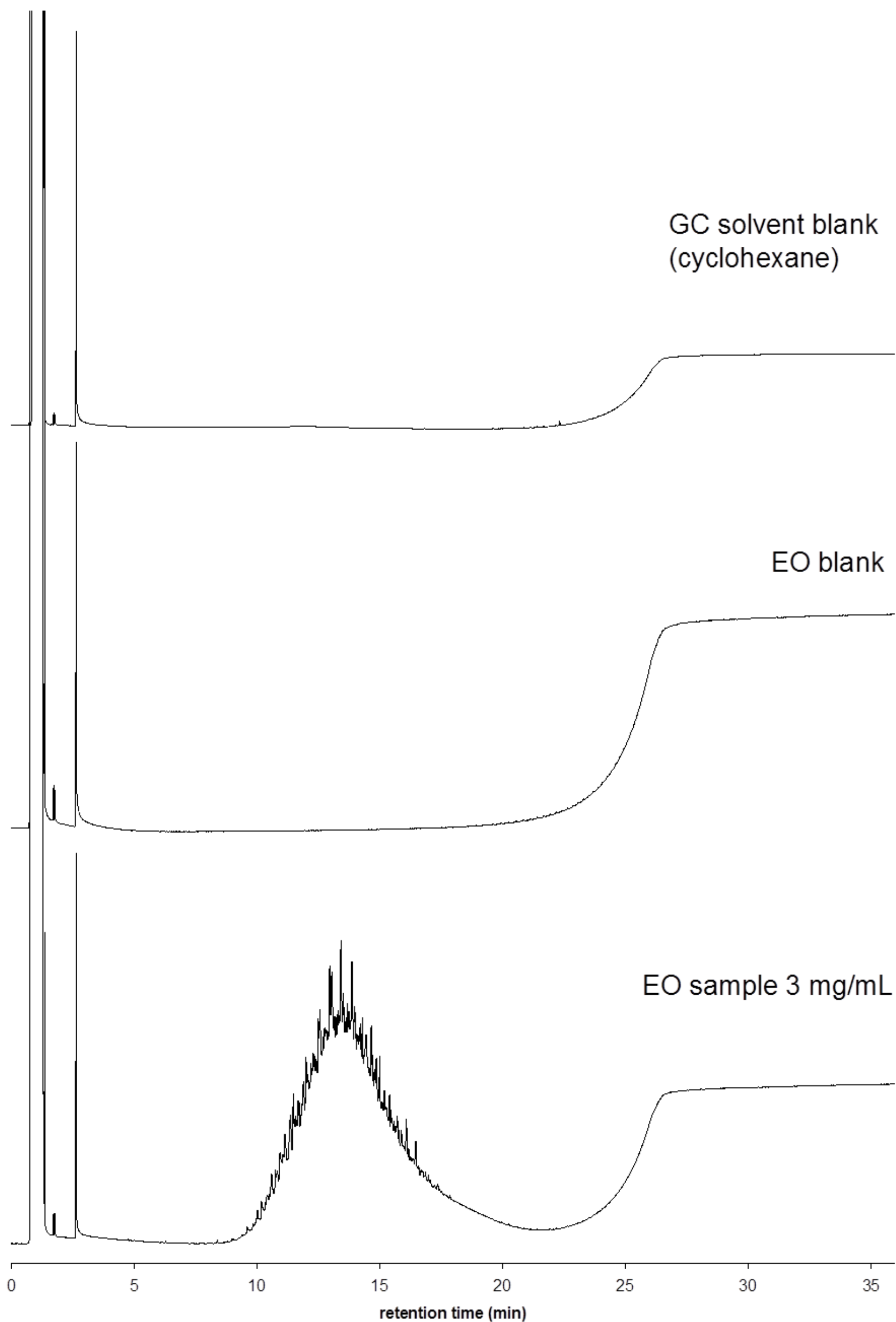
511 highlighting small proportions of C_2 to >C_8 alkylbenzenes and bibenzyl (latter from the
512 procedures used).



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516 **Figure S4** High temperature gas chromatograms (HTGC) for solvent (Top), procedural blank
517 (Middle) and 'saturates' fraction (Bottom) showing that there were no compounds eluting
518 above the retention time of ca. n-C_{40} alkane (ca. 20 minutes).
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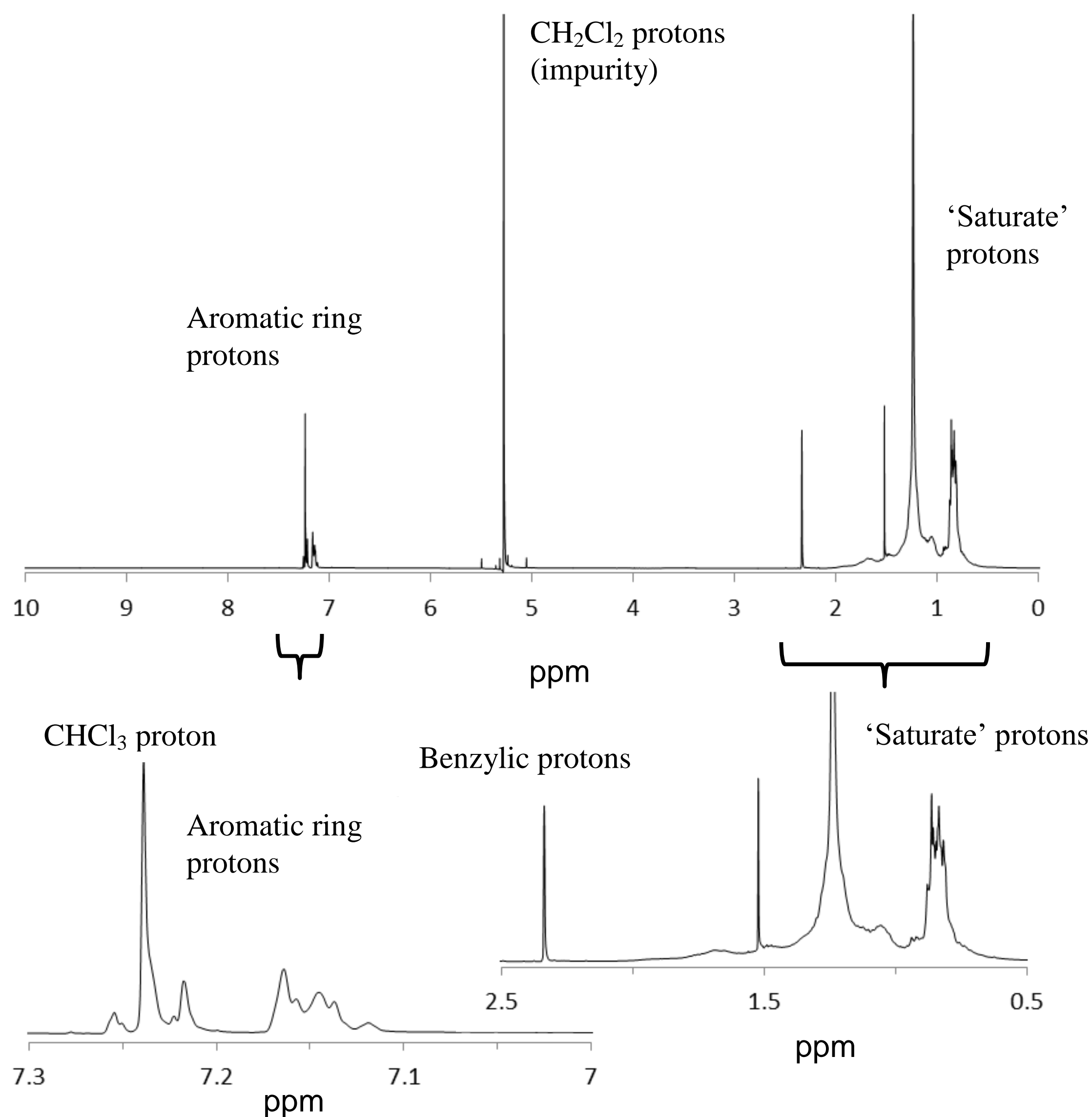

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524 **Figure S5** ^{1}H NMR spectrum of 'saturates' fraction dissolved in deuterated chloroform.
525

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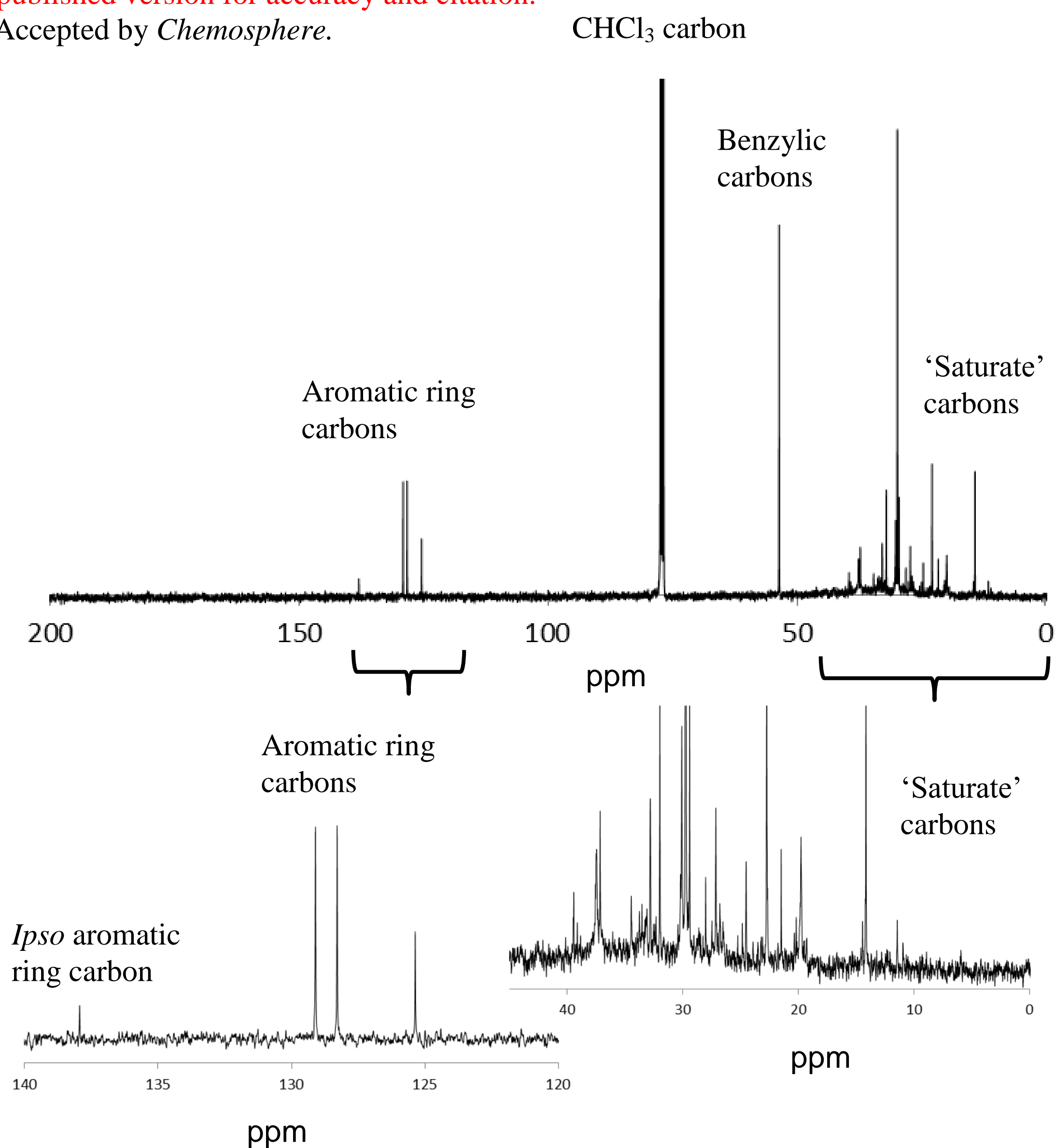

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531 **Figure S6** ^{13}C NMR spectrum of 'saturates' fraction dissolved in deuterated chloroform
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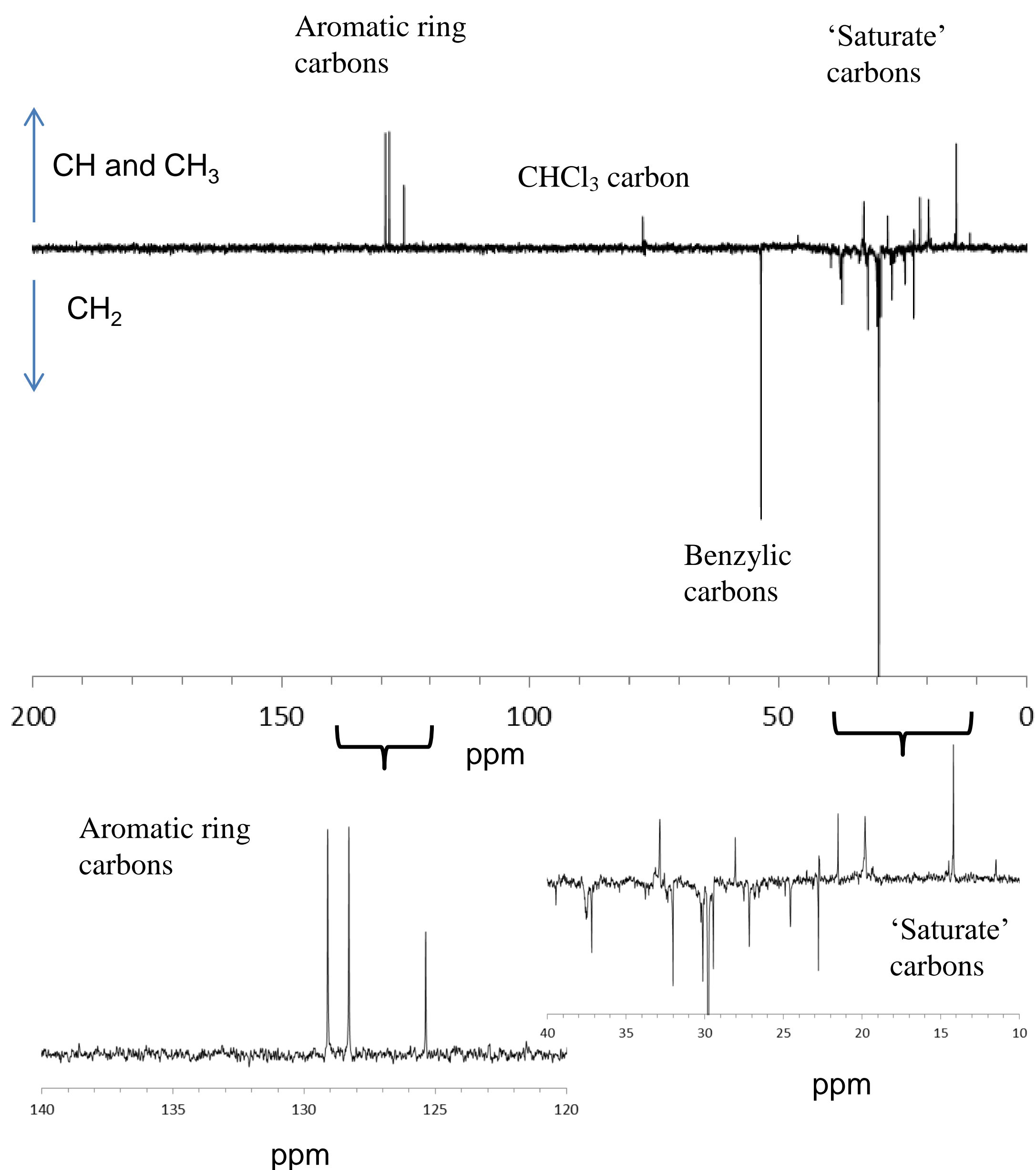

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538 **Figure S7** ^{13}C DEPT NMR spectrum of 'saturates' fraction dissolved in deuterated
539 chloroform
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