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1 Evidence of *in vitro* metabolic interaction effects of a chlorfenvinphos, ethion
2 and linuron mixture on human hepatic detoxification rates

3 Ali Kadar ^{a, b, *}, Georges de Sousa ^b, Ludovic Peyre ^b, Henri Wortham ^a, Pierre Doumenq ^a, Roger
4 Rahmani ^b.

5 ^a Aix Marseille Univ, CNRS, LCE, Marseille, France,

6 ^{*}Corresponding author. *E-mail address:* ali.kadar@etu.univ-amu.fr (A. Kadar)

7 ^b INRA, UMR 1331 TOXALIM, Laboratoire de Toxicologie Cellulaire et Moléculaire des
8 Xénobiotiques, BP 167, 400 Route des Chappes, 06903 Sophia Antipolis Cedex, France

9

10 ABSTRACT

11 General population exposure to pesticides mainly occurs via food and water consumption. However,
12 their risk assessment for regulatory purposes does not currently consider the actual co-exposure to
13 multiple substances. To address this concern, relevant experimental studies are needed to fill the lack
14 of data concerning effects of mixture on human health. For the first time, the present work evaluated
15 on human microsomes and liver cells the combined metabolic effects of, chlorfenvinphos, ethion and
16 linuron, three pesticides usually found in vegetables of the European Union. Concentrations of these
17 substances were measured during combined incubation experiments, thanks to a new analytical
18 methodology previously developed. The collected data allowed for calculation and comparison of the
19 intrinsic hepatic clearance of each pesticide from different combinations. Finally, the results showed
20 clear inhibitory effects, depending on the association of the chemicals at stake. The major metabolic
21 inhibitor observed was chlorfenvinphos. During co-incubation, it was able to decrease the intrinsic
22 clearance of both linuron and ethion. These latter also showed a potential for metabolic inhibition
23 mainly cytochrome P450-mediated in all cases. Here we demonstrated that human detoxification from
24 a pesticide may be severely hampered in case of co-occurrence of other pesticides, as it is the case for
25 drugs interactions, thus increasing the risk of adverse health effects. These results could contribute to

26 improve the current challenging risk assessment of human and animal dietary to environmental
27 chemical mixtures.

28

29 *Keywords:*

30 Health risk assessment, Pesticides mixture, Hepatic clearance, Metabolic interactions, Detoxification.

31

32 **1. Introduction**

33 Synthetic pesticides have helped to increase crop yields of modern agriculture for more than
34 half a century. However, due to their widespread use as insecticides, herbicides, fungicides, fumigants
35 and rodenticides, they are now considered as a major group of contaminants. For the general
36 population, although pesticide use for elimination of pests is a significant route of indoor exposure
37 ([Van den Berg et al., 2012](#)), dietary intake including water consumption is considered to be the main
38 source of exposure to most pesticides ([Cao et al., 2011](#); [Damalas et al., 2011](#); [Ding, 2014](#)). Thus, food
39 commodities may simultaneously contain different pesticide residues, resulting in an uninterrupted
40 exposure of human populations to complex pesticide mixtures through their diet. [Crepet et al. \(2013\)](#)
41 found that the French population is mainly exposed to 7 different pesticide mixtures composed of two
42 to six compounds (among 79 targeted food pesticides). As the marketing authorization for a chemical
43 substance is delivered at the European Union scale, it could be assumed that the whole European
44 population is likely to be exposed to these same pesticide mixtures. Among these residues, a mixture
45 including two organophosphorus compounds (chlorfenvinphos and ethion) banned since 2007 but not
46 necessarily totally off the agricultural practice ([Storck et al., 2017](#)) and a substituted urea (linuron),
47 was found to be frequently present in staple foods such as carrots and potatoes (see [Fig. 1](#)).

48 The organophosphorus insecticide chlorfenvinphos [2-chloro-1-(2,4-dichlorophenyl)vinyl
49 diethyl phosphate] is a neurotoxic molecule which inhibits the acetylcholinesterase. This phospho-
50 organic pesticide is transformed in mammals by a hepatic oxidative O-deethylation ([Hutson and](#)
51 [Wright, 1980](#)). Moreover, chlorfenvinphos administration leads to microsomal enzyme induction and
52 alterations of free amino acid concentrations in rat liver ([Sedrowicz et al., 1996](#)). In the same way, an

53 *in vivo* study has revealed that chlorfenvinphos decreases the glutathione level and increases the
54 concentrations of hydrogen peroxide and serum total glutathione in liver ([Lukaszewicz-Hussain,](#)
55 [2011](#)). Indeed, chlorfenvinphos liver metabolism is associated with cytochrome P450 (CYP) activities
56 resulting in the generation of reactive oxygenated metabolites and oxidative stress ([Swiercz et al.,](#)
57 [2013](#)).

58 Ethion (*O,O,O',O'*-tetraethyl *S,S'*-methylene bis(phosphoro-dithioate), is also an
59 organophosphorus insecticide, which presents the same mechanism of action, compared to
60 chlorfenvinphos. Ethion is converted in the liver to its active oxygenated analog, ethion mono-oxon,
61 *via* desulfuration thanks to cytochrome P-450 enzymes ([Desouky et al., 2013](#)).

62 Linuron, [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea] is a phenylurea herbicide widely
63 used in agriculture. Human liver is suspected to be a target of linuron, as it induced DNA damages in
64 rat liver ([Scassellati-Sforzolini et al., 1997](#)). Another study demonstrated that exposure to linuron leads
65 to hepatocellular adenomas in rat ([Santos et al., 2014](#)). Interestingly, this compound was described to
66 be activated in mammalian metabolizing cells leading to an increase of mutagenic properties ([Federico](#)
67 [et al., 2016](#)). Finally, linuron was shown to be an aryl hydrocarbon receptor (Ahr) ligand and its
68 agonistic activity leads to the induction of CYP1A genes' expression ([Takeuchi et al., 2008](#)).

69 Risk assessment carried out across the world by authorities mainly focus on compounds
70 belonging to the same chemical family, or possessing the same mechanisms of action ([Reffstrup et al.,](#)
71 [2010](#); [Ragas et al., 2013](#)). In addition, assessment is only based on the evaluation of cumulative effects
72 of these products, supposing the absence of potential effects concerning interactions between
73 pesticides ([European Food Safety Agency, 2012](#)). Therefore, a wide thinking process has been started
74 for more than 5 years to address the issue of risk assessment regarding the combined actions of
75 substances on human health ([Solecki et al., 2014](#); [European Commission, 2014](#); [Rider et al., 2013](#)). An
76 increasing number of experimental studies have been published in the last few years ([Starr et al., 2012](#);
77 [Takakura et al., 2013](#); [Carvalho et al., 2014](#); [Orton et al., 2014](#); [Cedergreen, 2014](#) [Clarke et al., 2015](#);
78 [Spaggiari et al., 2016](#)), helping to fill the gap in knowledge on this topic. More than a decade ago,
79 [Tang et al. \(2002\)](#) already demonstrated a strong inhibition in the metabolism of carbaryl when it was

80 simultaneously incubated with chlorpyrifos in human liver sub-cellular preparations. Similarly, Savary
81 et al. (2014) showed that hepatic metabolic interactions occurred during the co-incubation of the
82 pesticides endosulfan and methoxychlor. This phenomenon increases the residence time of the active
83 compounds and thus their latent toxicity. While Savary studied these interactions effects through the
84 activities of the CYP isoforms involved, Tang et al. (2002) used the same experimental strategy.
85 However, they also demonstrated the occurrence of an interaction effect on the basis of calculated
86 intrinsic clearance rates resulting from human liver microsome experiments. An alternative approach
87 to evaluate a metabolic interaction between two compounds consists in comparing the intrinsic
88 clearances of the product when incubated alone, or as a mixture using the substrate depletion approach
89 (Donglu et al., 2007).

90 Human liver is the most important site of biotransformation in the body, primary culture of
91 hepatocytes and hepatocyte subcellular preparations have proven to be suitable *in vitro* models for the
92 investigation of the metabolism and metabolic interactions of environmental contaminants (Hodgson
93 et al., 2014). Although liver microsomes support only a part of the whole metabolic process *i.e.* phase
94 I metabolism, it continues to be the first-line model for metabolism study assays. Indeed, they are
95 more readily available than hepatocytes and specifically adapted to CYP kinetic measurements. In
96 order to highlight the part of phase II metabolism and cellular uptakes, primary culture of hepatocytes
97 can be used as a complement as suggested by Houston and Carlile (1997), who demonstrated that both
98 microsomes and hepatocytes might be suitable for the ranking of specific substrate hepatic intrinsic
99 clearances in rats. Here, we investigated, for the first time, the effect of a co-incubation of multi-class
100 pesticides present as a mixture in the French diet, especially on the human liver metabolism. In order
101 to highlight the possible human metabolic interaction effects of the pesticide mixture, the analytical
102 method previously developed by Kadar et al. (2017) will be applied to this *in vitro* study.

103

104 **2. Materials and methods**

105 *2.1. Biological samples*

106 *2.1.1. Human microsomes*

107 The human hepatic microsomal preparation used was a pool of hepatic microsomes obtained
108 from ten different donors. First, for each individual, microsomes purification was carried out as
109 described by [Van der Hoeven and Coon \(1974\)](#). Then, for sample from each donor, the microsomal
110 protein concentration was quantified according to the method of Bradford (Bio-Rad Protein Assay kit;
111 Ref. 15), using bovine serum albumin as standard. Finally, a pooled microsomal sample was prepared
112 in order to obtain a final concentration of 10 mg/mL of proteins in 100 mM phosphate potassium
113 buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v). Aliquots of 1.4 mL were then
114 stored at -80 °C.

115 *2.1.2. Primary culture of human hepatocytes*

116 All experiments on human tissue were carried out according to the ethical standards of the
117 responsible committee on human experimentation and the Helsinki Declaration. For each of the three
118 livers donated (two females: F31 and F82, one male: M66), hepatocytes were isolated as previously
119 described by [Berry and Friend \(1969\)](#) and submitted to long-term cryopreservation ([De sousa et al.,](#)
120 [1991](#)). For the present study, they were thawed as established by [Rijntjes et al. \(1986\)](#). Viable cells
121 were suspended in seeding medium I (Williams E Glutamax medium supplemented with penicillin
122 100 UI/mL, streptomycin 100 µg/mL, bovine insulin 0.1 UI/mL and fetal calf serum 10% v/v). Cell
123 viability achieved by means of trypan blue exclusion was 80 % or greater, then the number of viable
124 cells was determined using a Malassez cell. The hepatocytes suspended in the seeding medium I were
125 inoculated after appropriate dilution at about 0.7×10^5 cells/well into 48-wells Corning® Costar®
126 plates (Corning, NY, USA) that had previously been coated with rat tail collagen I (Sigma Aldrich,
127 Saint-Quentin Fallavier, France). All the plates were then placed in an incubator with an atmosphere
128 containing 5 % of CO₂ and 95 % of relative humidity for a 24 h adhesion period. After attachment, the
129 wells were rinsed using medium II (Williams E Glutamax medium supplemented with penicillin
130 100 UI/mL, streptomycin 100 µg/mL, bovine insulin 0.1 UI/mL, hydrocortisone hemisuccinate 1 µM
131 and bovine serum albumin 240 µg/mL) and maintained in contact with the medium until the exposition
132 experiments.

133 2.2. *In vitro* metabolism experiments

134 2.2.1. *Microsomes incubations*

135 Microsomal samples at a total protein concentration of 0.5 mg/mL were prepared in 100 mM
136 phosphate buffer (pH 7.4) containing a cofactor regenerating system. This latter consisted of 6 mM
137 glucose-6-phosphate, 3 mM nicotinamide adenine dinucleotide phosphate and 0.4 unit of glucose-6-
138 phosphate dehydrogenase. Microsomal experiments were carried out in borosilicate glass tubes
139 (volume 6 mL) in order to minimize physical adsorption of the pesticides.

140 A limited volume of 1 μ L of stock individual solution of chlorfenvinphos, ethion, linuron or
141 their different combination mixtures, prepared in acetonitrile, was deposited in each tube. Then, after a
142 pre-incubation during 5 min at +37 °C, the microsomal sample was added. Care was taken in order not
143 to exceed a 0.25% solvent proportion in each tube. The blank sample was prepared using the same
144 volume of pure acetonitrile. The glass tube was incubated for the desired time as described above. At
145 the end of the experiments, the reactions were stopped by transferring tubes in an ice bath and by
146 adding 400 μ L of ice-cold acetonitrile to each tube before vortex stirring.

147 Firstly, for Michaelis–Menten experiments, independent compounds were incubated at +37°C
148 with the human microsomal sample in triplicate, at three increasing concentrations for chlorfenvinphos
149 (0.5, 1 and 2 μ M), ethion (1, 2 and 4 μ M) and linuron (3, 6 and 12 μ M). The reactions were stopped
150 after 4, 7, 11 and 21 min.

151 Secondly, on the base of the initial results, the Michaelis apparent affinity constant ($K_{m,app}$)
152 was determined for each xenobiotic as described in the data analysis part (see 2.3), and the metabolic
153 influence of co-incubations was studied. To achieve this goal, a single concentration of 0.2 $K_{m,app}$ of
154 each pesticide was incubated alone as a reference, with concentrations of 0.5 $K_{m,app}$ and 5 $K_{m,app}$ of
155 each other compounds and with both of them at the aforementioned levels. The assays carried out in
156 triplicate were quenched as described above at 3.5, 6.5 and 9.5 min.

157

158 2.2.2. Hepatocytes incubations

159 In each seeded well, the seeding medium was removed and substituted by 200 μ L of the same
160 medium supplemented with the required amount of the pesticides in order not to exceed 0.25 %
161 DMSO solvent proportion, and pre-incubated in the preparation glass tube at +37 °C during 5 min.
162 The blank seeded well consisted of the same percentage of pure DMSO solvent. After 5, 9, 15 or 22 h,
163 the hepatocytes were scraped and homogenized by a gentle manual agitation after immediate addition
164 of 200 μ L ice-cold acetonitrile.

165 First, to determine the Michaelis–Menten parameters, cells from three donors F31 (31-year-
166 old female), F82 (82-year-old female) and M66 (66-year-old male) were exposed to the pesticides at
167 four increasing concentrations of chlorfenvinphos (2, 4, 8, 12 μ M), ethion (4, 8, 12, 20 μ M) and
168 linuron (6, 12, 18, 24 μ M). All the reactions were stopped after 5, 9, 15 and 22 h as reported above.
169 Secondly, after determination of the Michaelis apparent affinity constant ($K_{m,app}$) of each pesticide, the
170 effect of co-incubation on individual metabolism was studied. To realize this aim, a single
171 concentration of 0.1 $K_{m,app}$ of each pesticide was incubated alone as a reference, with concentrations of
172 0.1 $K_{m,app}$ and 0.5 $K_{m,app}$ of each other compounds and with both of them at the same last two levels.
173 Each experiment was performed in triplicate with pools of hepatocytes from the three individuals
174 mentioned above (*i.e.* pooled after thawing) in order to represent an average human donor pool.
175 Consequently, the pesticides dependent $K_{m,app}$ values were calculated as the average of the specific
176 constants of each individual. The incubations were finalized after 5, 10 and 15 h as described above.

177 2.3. Data analysis

178 Michaelis–Menten affinity constants ($K_{m,app}$) and rates of intrinsic clearance (CL_{int}) were
179 calculated from parent compound depletion data. For $K_{m,app}$ determination, concentration of pesticide
180 remaining over the time course was determined thanks to the analytical method presented by [Kadar et](#)
181 [al. \(2017\)](#). Moles of pesticide remaining were then converted into moles of product formed and,
182 plotted versus time to allow determination of the kinetic reaction after nonlinear least square
183 regression analysis using GraphPad Prism6 software (Ritme, Paris, France). In order to reduce the

184 number of compound concentrations needed to accurately determine Michaelis-Menten kinetic
185 parameters, the alternative direct-linear plot approach (Eisenthal and Cornish-Bowden, 1974) was
186 employed. According to this method, for each pesticide, only two to four pesticide concentrations
187 were needed to calculate $K_{m,app}$. Finally, intrinsic clearances were determined directly using the *in vitro*
188 half-life method (Obach, 1999). A regression analysis was carried out to determine $T_{1/2}$ value, before a
189 conversion to CL_{int} as described by the formulas (1) to (3) presented below.

190

$$191 \quad S = S_0 \times e^{-kt} \quad (1)$$

192 Where S (μM) is the remaining substrate (pesticide) concentration, S_0 the initial substrate
193 concentration (μM) and k the elimination rate constant (min^{-1} or h^{-1})

194

$$195 \quad T_{1/2} = \frac{-0.693}{k} \quad (2)$$

196 Where $T_{1/2}$ is the half-life value for respectively microsomes (min) and hepatocytes assays (h)

197

$$198 \quad CL_{int} = \frac{0.693}{T_{1/2}} \times \frac{V_i}{Q} \quad (3)$$

199 Where V_i is the incubation volume (mL) and Q the proteins amount (mg)

200

201 **3. Results and discussion**

202 *3.1. Microsomes metabolism study*

203 The *in vitro* metabolism of each pesticide was first investigated using human liver
204 microsomes. Incubation mixtures of each target analyte displayed no biotransformation when
205 experiments were performed in the absence of a NADPH-generating system, implying a CYP
206 dependent metabolism. As shown in Fig. 2 and summarized in Table 1, the $K_{m,app}$ values obtained after
207 data processing for each pesticide were, $4.2 \pm 0.3 \mu\text{M}$, $8.0 \pm 0.3 \mu\text{M}$ and $2.0 \pm 0.1 \mu\text{M}$ for
208 chlorfenvinphos, ethion and linuron respectively.

209 As described by Obach (1999), we assumed that an approximate value of $0.2 K_{m,app}$ was well
210 below the $K_{m,app}$. Then, level values of $0.4 \mu\text{M}$ for linuron, $0.8 \mu\text{M}$ for chlorfenvinphos and $1.6 \mu\text{M}$ for
211 ethion should be on the part of the saturation curve where initial rates correlate with CL_{int} . The *in vitro*
212 half-life data collected from individual and simultaneously incubated pesticides allowed the final
213 calculation of the hepatic intrinsic clearances displayed in Table 2.

214 Firstly, the human liver microsomal intrinsic clearance of linuron pesticide
215 ($0.681 \text{ mL}/\text{min}/\text{mg}$) was not clearly impaired when the lowest concentrations of either
216 chlorfenvinphos ($2 \mu\text{M}$) or ethion ($4 \mu\text{M}$) were individually added. Indeed, their respective intrinsic
217 clearances were 0.678 and $0.672 \text{ mL}/\text{min}/\text{mg}$, which was not noticeably different from the linuron's
218 intrinsic clearance. However, their joint-combination at the same low concentrations revealed an
219 important decrease of the linuron intrinsic clearance ($0.499 \text{ mL}/\text{min}/\text{mg}$). In addition, ethion co-
220 exposure at $40 \mu\text{M}$ slightly changed the intrinsic clearance, dividing it by a 1.1 fold. On the contrary,
221 the presence of chlorfenvinphos at $20 \mu\text{M}$ decreased by a 2.7 fold linuron's biotransformation. An
222 interesting synergistic effect could be noticed when linuron was co-incubated with a mixture of ethion
223 and chlorfenvinphos at their highest levels, leading to a 3.7 fold drop off of the intrinsic linuron's
224 intrinsic clearance.

225 Chlorfenvinphos intrinsic clearance was $1.069 \text{ mL}/\text{min}/\text{mg}$ when the pesticide was incubated
226 alone. Low amounts of either linuron or ethion did not clearly influence its intrinsic clearance.
227 However, we observed a remarkable effect on the original intrinsic clearance when chlorfenvinphos
228 was individually co-incubated with the highest amounts of linuron ($10 \mu\text{M}$) or ethion ($40 \mu\text{M}$). Indeed,
229 in each condition, we noticed a decrease of approximately 1.5 and 1.4-fold, respectively. Moreover,
230 the presence of a mixture containing these two inhibitors at their highest levels induced a comparable
231 division (1.5 fold) of chlorfenvinphos' intrinsic clearance, suggesting that a competitive interaction
232 was very likely to occur in this case.

233 Finally, in the third and last experimental conditions, ethion's intrinsic clearance
234 ($0.802 \text{ mL}/\text{min}/\text{mg}$) was not clearly modulated by the presence of linuron, whatever the concentration
235 level. On the other hand, when co-incubated with chlorfenvinphos at its low level, ethion's intrinsic

236 clearance was divided by a 1.3-fold and, at its highest level by a 2.7-fold. The decrease reached a 3.5-
237 fold when ethion was incubated with a mixture of the two other pesticides at their highest levels.

238 As a consequence, these results obtained from the microsomes metabolism study showed that
239 even if each compound of the studied mixture revealed inhibiting actions, chlorfenvinphos was the
240 most potent inhibitor. Added separately to linuron or ethion, it greatly inhibited the metabolization of
241 these two pesticides with intrinsic clearance values close to those observed in the mixture exposition
242 conditions. Moreover, as weaker inhibitors, linuron and ethion failed to exert such inhibitory effect
243 when they were in turn added to chlorfenvinphos. This suggested once again the predominant role of
244 chlorfenvinphos in the inhibition of the hepatic intrinsic clearance.

245 3.2. Hepatocytes metabolism study

246 The second part of the study enabled to examine the *in vitro* metabolism of each phytosanitary
247 product, using primary cultures of human hepatocytes. This step allowed evaluating whether the
248 mixture interactions revealed above could be confirmed with the use of the considered “gold model”,
249 knowing that freshly isolated hepatocytes, express all the hepatic enzymes and transporters required
250 for complete metabolism studies (Fasinu et al., 2012).

251 Firstly, as depicted in Fig. 3 and summarized in Table 1, the $K_{m,app}$ values of each pesticide
252 were estimated for the different liver cells. We found that the three donors presented a 2 to 3-fold
253 inter-individual variability, while F82 exhibited the highest affinity values (*i.e.* lowest values for K_m),
254 followed by M66 and F31. The average $K_{m,app}$ values were, $42.2 \pm 24.8 \mu\text{M}$, $43.0 \pm 14.1 \mu\text{M}$ and
255 $20.0 \pm 13.2 \mu\text{M}$ for chlorfenvinphos, ethion and linuron, respectively. Concentrations of $2.0 \mu\text{M}$ for
256 linuron, $4.0 \mu\text{M}$ for chlorfenvinphos and also $4.0 \mu\text{M}$ for ethion were chosen greatly below the $K_{m,app}$.
257 so as to be located in the range where each individual intrinsic clearance is constant, as presented
258 above in the microsomes model. The data obtained from individually and simultaneously exposed
259 pesticides are summarized in Table 3.

260 The human hepatocyte intrinsic clearance of chlorfenvinphos was 5.25 mL/h/mg . When co-
261 exposed with the highest dose of either linuron or ethion, the intrinsic clearance was reduced until 3.69
262 and 4.18 mL/h/mg respectively. The presence of ethion and linuron at low concentrations produced a

263 noticeable but limited effect, whereas a strong inhibition was observed at high concentrations resulting
264 in a 1.7-fold decrease of the chlorfenvinphos intrinsic clearance.

265 When incubated alone, ethion's intrinsic clearance was 5.07 mL/h/mg. Even if a slight
266 inhibitory effect appeared with a decrease of this intrinsic clearance until approximately 4.70 mL/h/mg
267 when chlorfenvinphos and linuron were individually co-incubated at 10 μ M or 4 μ M respectively, a
268 more pronounced decrease of about a 1.1-fold, was observed when these two pesticides were present
269 as a mixture at their lowest concentrations. Moreover, chlorfenvinphos had a more important influence
270 when it was co-incubated at its highest concentration (20 μ M) with ethion, decreasing the ethion's
271 intrinsic clearance by a 1.3-fold. The concomitance of ethion with the highest levels of a mixture of
272 chlorfenvinphos and linuron lead to reach a 1.7-fold reduction in its hepatic detoxification.

273 Linuron intrinsic clearance was 2.95 mL/h/mg when exposed alone at 2.0 μ M to hepatocytes
274 cells. This value remained unchanged when the lowest amounts of ethion or chlorfenvinphos were also
275 present. However, as for the precedent pesticide, a slight decrease of about 1.1-fold was calculated
276 when these substances were both added to the cellular medium at their lowest concentrations. This
277 decrease of the clearance also reached an approximate 1.3-fold at the highest chlorfenvinphos
278 concentration (20 μ M). Lastly, linuron's intrinsic clearance was divided by about a 1.8-fold when this
279 pesticide was incubated in the presence of a mixture of the two other compounds at their highest
280 concentrations (20 μ M).

281 Thus, the observations made during the hepatocytes metabolism study showed numerous
282 similarities with the microsomal experiments presented above. Indeed, even if each compound of the
283 studied mixture showed a capacity to hamper the degradation of the other pesticides, chlorfenvinphos
284 was the strongest inhibitor among the three pesticides under study. Overall, the co-incubations of the
285 studied compounds on both microsomes and hepatocytes depicted similar metabolic effects. We thus
286 suggest that the intrinsic clearance inhibitions could be mainly due to the inhibition of phase I
287 metabolic enzymes, with minor involvement of other cellular enzymes and membrane transporters.
288 Indeed, herbicides from phenylurea family are known to be mainly metabolized by human CYP1A2,
289 CYP2C19 and CYP3A4 enzyme isoforms and also, but less importantly, by CYP2B6 ([Abass et al.](#),

290 2007). On the other hand, human organophosphorus metabolism is mainly CYP1A2, CYP2B6,
291 CYP2C9, CYP2C19 and CYP3A4 mediated (Hodgson et al., 2007). As a consequence, this confirms
292 that chlorfenvinphos, ethion and linuron share a common support by CYP for their detoxification and,
293 are likely to compete for the same active sites, resulting in the decrease of their respective intrinsic
294 clearance as experimentally observed above. Moreover, among these phase I enzymes, CYP1A2,
295 CYP2B6 and CYP3A4 are known to be most active in the formation of active oxon phosphate
296 metabolites (Buratti et al., 2004). During this activation by oxidative desulfuration reaction, the release
297 of highly reactive sulfur results in irreversible CYP inhibitions (Hodgson et al., 2007). This is
298 precisely what could happen with ethion but not with chlorfenvinphos which, in contrast to most
299 organophosphorus pesticides, presents an already desulfurated active parent form (Carter et al., 2010)
300 as shown by its typical oxon chemical structure (Fig. 1). Therefore, the potent inhibitory characteristic
301 shown by this compound may be explained through the action of other enzymes. Furthermore, the
302 noticeable difference in intrinsic clearances between human liver models mentioned above might be
303 due to the faster metabolization of chlorfenvinphos in cells. Indeed, as an oxon, the most potent
304 inhibitor of the pesticide mixture should be more easily metabolized through the hydrolytic action of
305 hepatic paraoxonase, which is far less present in microsomes than in hepatocytes (Gonzalvo et al.,
306 1997).

307

308 4. Conclusions

309 In this work, a metabolism study on chlorfenvinphos, ethion and linuron mixture was
310 conducted. It aimed at exploring the possible pesticide-pesticide(s) human liver metabolic interactions
311 through the monitoring of the *in vitro* loss of the parent compound. A minimized number of assays
312 provided the Michaelis-Menten $K_{m,app}$ parameter for each compound after Eisenthal and Cornish-
313 Bowden velocities plotting. Based on combined incubations of the active substances, interaction
314 experiments showed clear inhibition effects in both human liver microsomes and hepatocytes. Even if
315 all the compounds showed an action, the rank order of the individual inhibitory potency was
316 chlorfenvinphos >> linuron > ethion. In liver microsomes, the major metabolic inhibitions were

317 observed after ethion and linuron concomitant treatments, with a high level of the two other products
318 displaying respectively a 3.5 and 3.7 fold reduction of their intrinsic clearance. These inhibitions were
319 also observed in primary cultures of hepatocytes but were reduced by approximately a half. We
320 hypothesized that it might be linked to a decrease in chlorfenvinphos' inhibitory strength, due to the
321 presence in the liver cells of much greater paraoxonase amounts.

322 Moreover, the similar trends revealed by the two human liver models demonstrated that the
323 metabolic interactions were mainly mediated by phase I enzymes, probably CYP1A2, CYP2C19,
324 CYP3A4 and CYP2B6.

325 To conclude, we found evidence of *in vitro* metabolic interaction effects of a chlorfenvinphos,
326 ethion and linuron mixture on human hepatic detoxification rates. As the consumption of vegetables
327 such as potatoes or carrots proved to have an impact on hepatic intrinsic clearances of these pesticides,
328 we can hypothesize that in France, at the European scale and very probably also abroad, due to a delay
329 in the detoxification of the body, people may be exposed to an increased toxicity of these pesticides.
330 Both experiments using CYP isoforms and paraoxonase, and a comprehensive study focusing on
331 additivity, synergism, and antagonism interactions of the studied mixture should be useful. The results
332 of this work may already encourage safety agencies to include the issue of pesticide mixtures in
333 dietary and environmental risk assessment processes. The methodology described in this work could
334 be applied for future studies on harmful chemical mixtures present in humans and animals' diets, as
335 well as in their environment.

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343 **References**

- 344 Berry, M.N., Friend, D.S, 1969. High-yield preparation of isolated rat liver parenchymal cells: a
345 biochemical and fine structural study. *J. Cell Biol.* 43, 506–20.
- 346 Cao, L.L., Yan, C.H., Yu, X.D., Tian, Y., Zhao, L., Liu, J.X., Shen, X.M., 2011. Relationship between
347 serum concentrations of polychlorinated biphenyls and organochlorine pesticides and dietary habits of
348 pregnant women in Shanghai. *Sci. Total Environ.* 409, 2997–3002.
- 349 Carlile, D.J., Hakooz, N., Bayliss, M.K., Houston, J.B., 1999. Microsomal prediction of in vivo
350 clearance of CYP2C9 substrates in humans. *Br. J. Clin. Pharmacol.* 47, 625–635.
- 351 Carter W.G., Tarhoni M.H., Ray D.E., 2010. Analytical approaches to investigate protein-pesticide
352 adduct. *J Chromatogr B Analyt Technol Biomed Life Sci.* 878:1312-1319.
- 353 Carvalho, R.N., Arukwe, A., Ait-Aissa, S., Bado-Nilles, A., Balzamo, S., Baun, A., Belkin, S. et al.,
354 2014. Mixtures of chemical pollutants at European legislation safety concentrations: how safe are
355 they? *Toxicol. Sci.* 141, 218–233.
- 356 Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within
357 environmental toxicology. *PLoS One.* 9, 1–12.
- 358 Clarke, R., Connolly, L., Frizzell, C., Elliott, C.T., 2015. Challenging conventional risk assessment
359 with respect to human exposure to multiple food contaminants in food: A case study using maize.
360 *Toxicol. Lett.* 238, 54–64.
- 361 Damalas, C.A., Eleftherohorinos, I.G., 2011. Pesticide Exposure, Safety Issues, and Risk Assessment
362 Indicators. *Int. J. Environ. Res. Public Health.* 8, 1402–1419.
- 363 Desouky M.M., Abdel-Gawad H., Hegazi B., 2013. Distribution, fate and histopathological effects of
364 ethion insecticide on selected organs of the crayfish, *Procambarus clarkii*. *Food Chem. Toxicol.* 52,
365 42-52.

366 De Sousa, G., Dou, M., Barbe, D., Lacarelle, B., Placidi, M., Rahmani, R., 1991. Freshly isolated or
367 cryopreserved human hepatocytes in primary culture: Influence of drug metabolism on hepatotoxicity.
368 *Toxicol. In Vitro* 5, 483–486.

369 Ding, G., 2014. Revisiting pesticide exposure and children's health: focus on China. *Sci. Total*
370 *Environ.* 472, 289–295.

371 Donglu, Z, Mingshe, Z., Griffith Humphreys, W., 2007. *Drug Metabolism in Drug Design and*
372 *Development: Basic Concepts and Practice*
373 <http://onlinelibrary.wiley.com/doi/10.1002/9780470191699.fmatter/pdf>.

374 Eisenthal, R., Cornish-Bowden, A., 1974. The direct linear plot. A new graphical procedure for
375 estimating enzyme kinetic parameters. *Biochem. J.* 139, 715–20.

376 European Commission (2014) *Assessment of Mixtures-Review of Regulatory Requirements and*
377 *Guidance.* <<https://ec.europa.eu>> (Accessed 5 June 2015).

378 European Food Safety Agency (2012). *Scientific Report on Investigation of the state of the science on*
379 *combined actions of chemicals in food through dissimilar modes of action and proposal for science-*
380 *based approach for performing related cumulative risk assessment.* Available from
381 <www.efsa.europa.eu> (Accessed 5 June 2015).

382 Federico, C., Palmieri, C., Pappalardo, A.M., Ferrito, V., Pappalardo, M., Librando V., Saccone, S.,
383 2016. Mutagenic properties of linuron and chlorbromuron evaluated by means of cytogenetic
384 biomarkers in mammalian cell lines. *Environ Sci Pollut Res Int.* 23, 17018-25.

385 Hodgson, E., Wallace, A.D., Shah, R.R., Choi, K., Joo, H., 2014. *Human Variation and Risk*
386 *Assessment: Microarray and Other Studies Utilizing Human Hepatocytes and Human Liver*
387 *Subcellular Preparations.* *J. Biochem. Mol. Toxicol.* 1, 1–10.

388 Hodgson, E. and Rose R.L., 2007. Human metabolic interactions of environmental chemicals.
389 *J. Biochem. Mol. Toxicol.* 21: 182–6.

390 Houston, J.B., Carlile, D.J., 1997. Prediction of hepatic clearance from microsomes, hepatocytes and
391 liver slices. *Drug Metab. Rev.* 29, 891–922.

392 Lukaszewicz-Hussain, A., 2011. Liver and serum glutathione concentration and liver hydrogen
393 peroxide in rats subchronically intoxicated with chlorfenvinphos--organophosphate insecticide. *Med*
394 *Pr.* 62, 23-9.

395 Obach, R.S., 1999. Prediction of human clearance of twenty-nine drugs from hepatic microsomal
396 intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to
397 microsomes. *Drug Metab. Dispos.* 27, 1350–9.

398 Orton, F., Ermler, S., Kugathas, S., Rosivatz, E., Scholze, M., Kortenkamp, A., 2014. Mixture effects
399 at very low doses with combinations of anti-androgenic pesticides, antioxidants, industrial pollutant
400 and chemicals used in personal care products. *Toxicol. Appl. Pharmacol.* 278, 201–8.

401 Ragas, A.M., Holmstrup, M., 2013. Tools and perspectives for assessing chemical mixtures and
402 multiple stressors. *Toxicology.* 313, 73–82.

403 Reffstrup, T.K., Larsen, J.C., Meyer, O., 2010. Risk assessment of mixtures of pesticides. *Current*
404 *approaches and future strategies. Regul. Toxicol. Pharmacol.* 56, 174–192.

405 Rider, C.V., Carlin, D.J., De Vitoa, M.J., Thompson, C.L., Walkera, N.J., 2013. Mixtures research at
406 NIEHS: An evolving program. *Toxicology.* 313, 94–102.

407 Santos, S.M., Videira, R.A., Fernandes, M.A., Santos, M.S., Moreno, A.J., Vicente, J.A., Jurado, A.S.,
408 2014. Toxicity of the herbicide linuron as assessed by bacterial and mitochondrial model systems.
409 *Toxicol In Vitro.* 28, 932-9.

410 Savary, C.C, Jossé, R., Bruyère, A., Guillet, F., Robin, M.A., Guillouzo, A., 2014. Interactions of
411 endosulfan and methoxychlor involving CYP3A4 and CYP2B6 in human HepaRG cells. *Drug Metab.*
412 *Dispos.* 42, 1235–1240.

413 Scassellati-Sforzolini, G., Pasquini, R., Moretti, M., Villarini, M., Fatigoni, C., Dolara, P., Monarca,
414 S., Caderni, G., Kuchenmeister, F., Schmezer, P., Pool-Zobel, B.L., 1997. In vivo studies on
415 genotoxicity of pure and commercial linuron. *Mutat Res.* 390, 207-21.

416 Sedrowicz, L., Witkowska, D., Oledzka, R., 1996. Effect of chlorfenvinphos, cypermethrin and their
417 mixture on the intestinal transport of leucine and methionine. *J. Appl. Toxicol.* 16, 483-9.

418 Solecki, R., Stein, B., Frische, T., Matezki S, Wogram J, Streloke M., 2014. Paradigm shift in the risk
419 assessment of cumulative effects of pesticide mixtures and multiple residues to humans and wildlife:
420 German proposal for a new approach. *J. Verbr. Lebensm.* 9, 329–31.

421 Spaggiari D., Daali Y., Rudaz S., 2016. An extensive cocktail approach for rapid risk assessment of in
422 vitro CYP450 direct reversible inhibition by xenobiotic exposure. *Toxicol Appl Pharmacol.*

423 Starr, J.M., Scollon, E.J., Hughes, M.F., Ross, D.G., Graham, S.E., Crofton, K.M., et al., 2012.
424 Environmentally relevant mixtures in cumulative assessments: an acute study of toxicokinetics and
425 effects on motor activity in rats exposed to a mixture of pyrethroids. *Toxicol. Sci.* 130, 309–18.

426 Storck V., Karpouzas D. G., Martin-Laurent F., 2017. Towards a better pesticide policy for the
427 European Union. *Sci. Total Environ.* 575, 1027–1033.

428 Swiercz, R., Lutz P., Gralewicz, S., Grzelińska, Z., Piasecka-Zelga, J., Wąsowicz, W., 2013. Partial
429 protection from organophosphate-induced cholinesterase inhibition by metyrapone treatment. *Int J*
430 *Occup Med Environ Health.* 26, 636-46.

431 Takakura, N., Sanders, P., Fessard, V., Le Hégarat, L., 2013. In vitro combined cytotoxic effects of
432 pesticide cocktails simultaneously found in the French diet. *Food Chem. Toxicol.* 52, 153–62.

433 Takeuchi, S., Iida, M, Yabushita, H., Matsuda, T., Kojima, H., 2008. In vitro screening for aryl
434 hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line,
435 DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and
436 linuron. *Chemosphere.* 74, 155-65.

- 437 Tang, J., Cao, Y., Rose, R.L., Hodgson, E., 2002. In vitro metabolism of carbaryl by human
438 cytochrome P450 and its inhibition by chlorpyrifos. *Chem-Biol. Interact.* 141, 229–241.
- 439 Van den Berg, H., Zaim, M., Mnzava, A., Hii, J., Dash, A.P., Ejov, M., 2012. Global trends in the use
440 of insecticides to control vector-borne diseases. *Environ. Health Perspect.* 120, 577–82.
- 441 Van der Hoeven, T.A., Coon, M.J., 1974. Preparation and properties of partially purified cytochrome
442 P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase from
443 rabbit liver microsomes. *J. Biol. Chem.* 249, 6302–10.

Table 1

Michaelis apparent affinity constant values from human microsomes and hepatocytes for chlorfenvinphos, ethion and linuron

Pesticide	$K_{m,app}$ (μM)				
	Microsomes		Hepatocytes		
	*Pool (10 donors, n=3)	M66	F31	F82	*Average (3 donors)
Chlorfenvinphos	4.2±0.3	44.9	65.5	16.2	42.2±25.8
Ethion	8.0±0.6	42.4	57.3	29.2	43.0±14.1
Linuron	2.0±0.1	14.1	35.2	10.8	20.0±13.2

* ± S.D.

Table 2

Effects of co-incubations on individual human liver microsomes intrinsic clearance

Target clearance	incubated pesticide(s)						
	–	ethion		chlorfenvinphos		mixture	
Pesticide concentrations (μM)	–	4	40	2	20	4+2	40+20
*Cl _{int} <i>linuron</i>	0.681	0.678	0.620	0.672	0.248	0.499	0.186
	–	linuron		ethion		mixture	
Pesticide concentrations (μM)	–	1	10	4	40	1+4	10+40
*Cl _{int} <i>chlorfenvinphos</i>	1.069	1.020	0.714	1.067	0.764	0.984	0.702
	–	linuron		chlorfenvinphos		mixture	
Pesticide concentrations (μM)	–	1	10	2	20	1+2	10+20
*Cl _{int} <i>ethion</i>	0.802	0.767	0.748	0.623	0.292	0.593	0.227

* expressed in mL/min/mg of human hepatic microsomes

Table 3

Effects of co-incubations on intrinsic clearance of individual human liver hepatocytes.

Target clearance	incubated pesticide(s)						
	–	ethion		chlorfenvinphos		mixture	
Pesticide concentrations (μM)	–	4	20	4	20	4+4	20+20
*Cl _{int} <i>linuron</i>	2.951	2.942	2.799	2.825	2.362	2.666	1.629
	–	linuron		ethion		mixture	
Pesticide concentrations (μM)	–	2	10	4	20	2+4	10+20
*Cl _{int} <i>chlorfenvinphos</i>	5.250	5.084	3.688	5.215	4.174	4.9940	2.834
	–	linuron		chlorfenvinphos		mixture	
Pesticide concentrations (μM)	–	2	10	4	20	2+4	10+20
*Cl _{int} <i>ethion</i>	5.067	4.827	4.707	4.725	3.890	4.576	2.965

* expressed in mL/h/mg of human hepatic proteins, 50 mg proteins per gm of liver (Carlile et al., 1999).

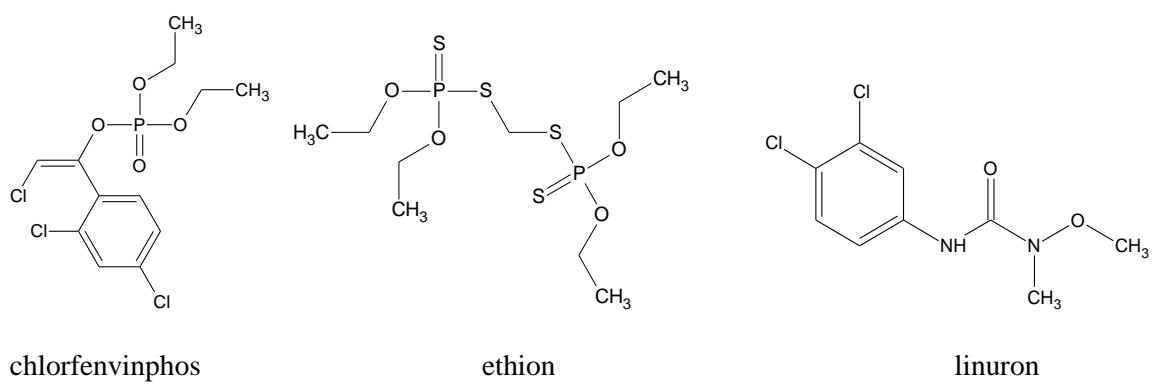
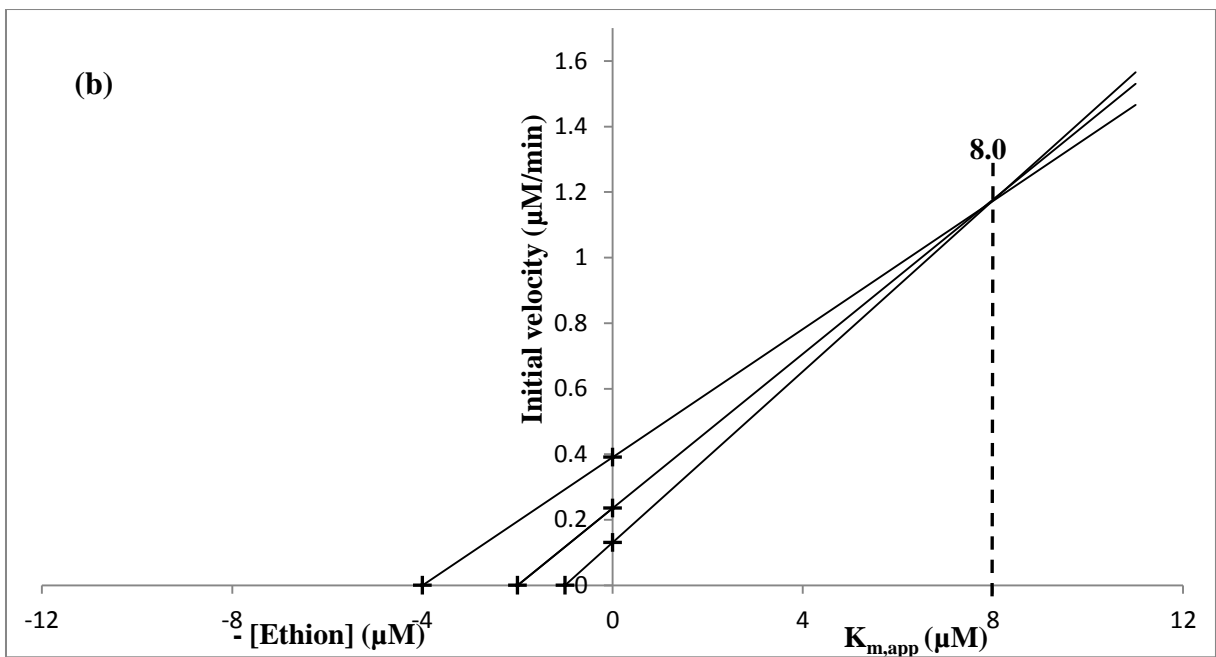
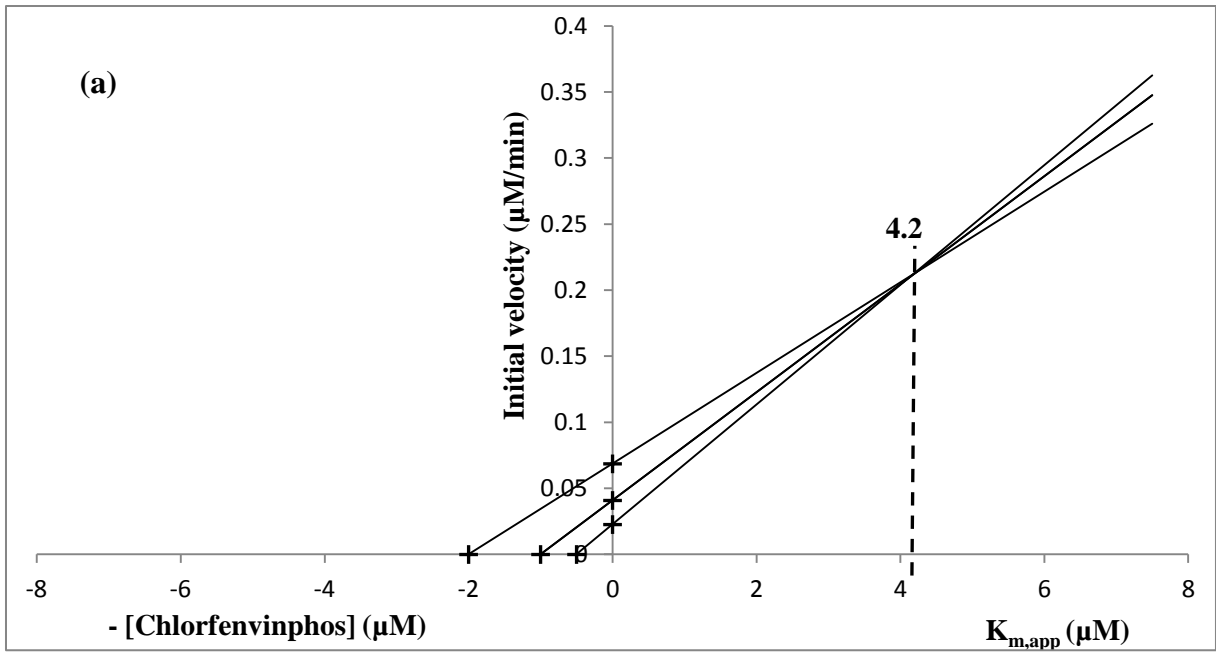


Fig. 1. Molecular structures of chlorfenvinphos, ethion and linuron



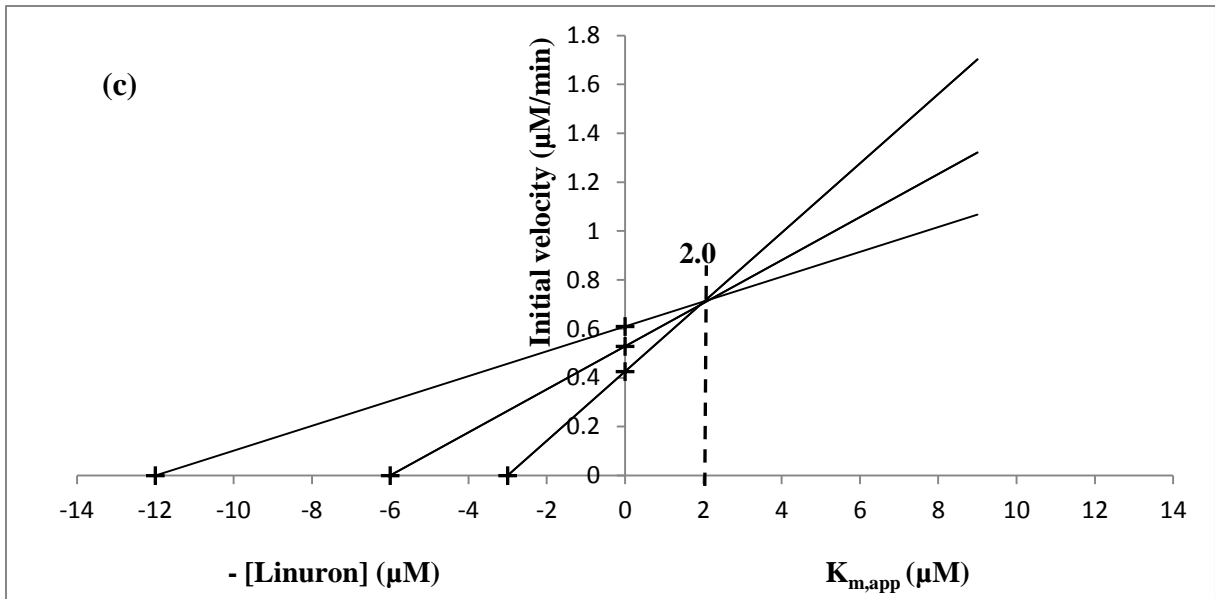
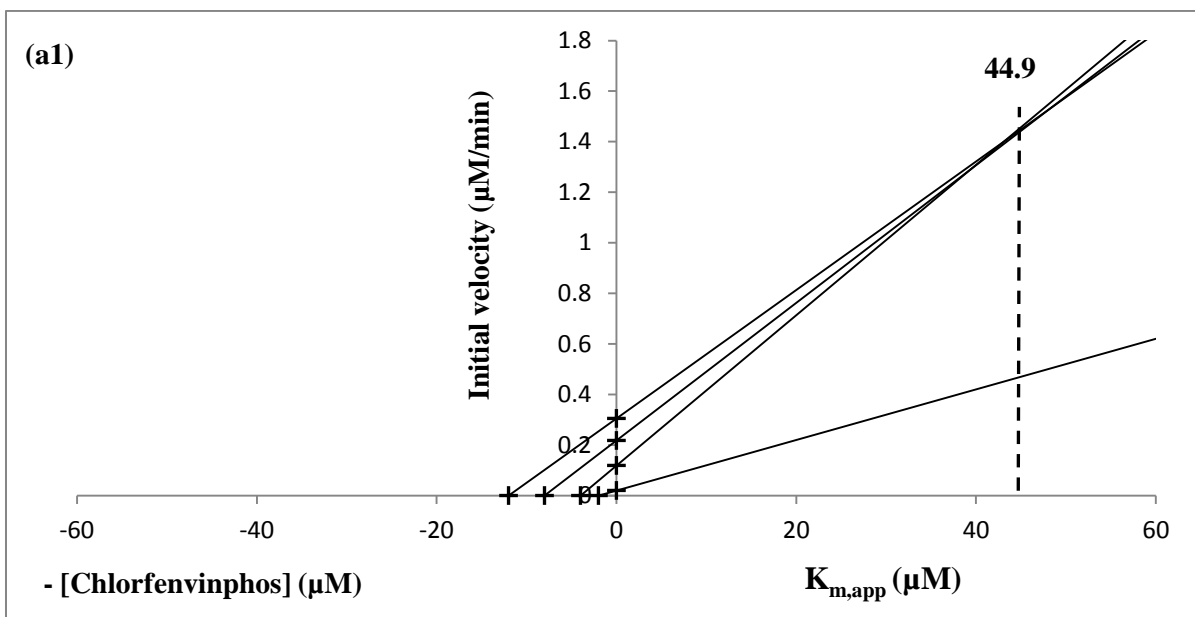
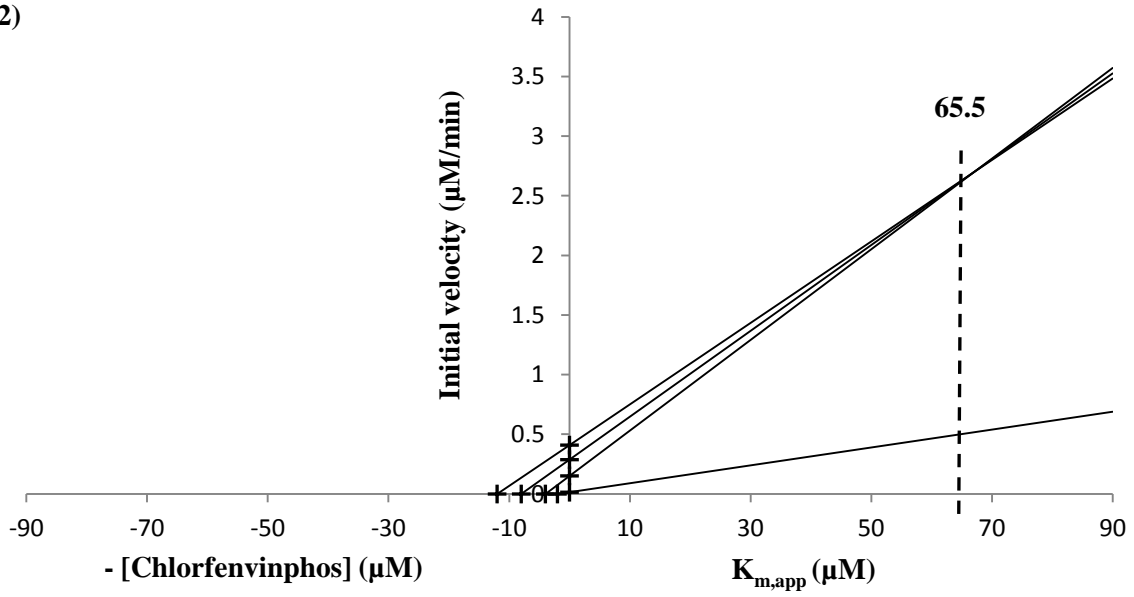


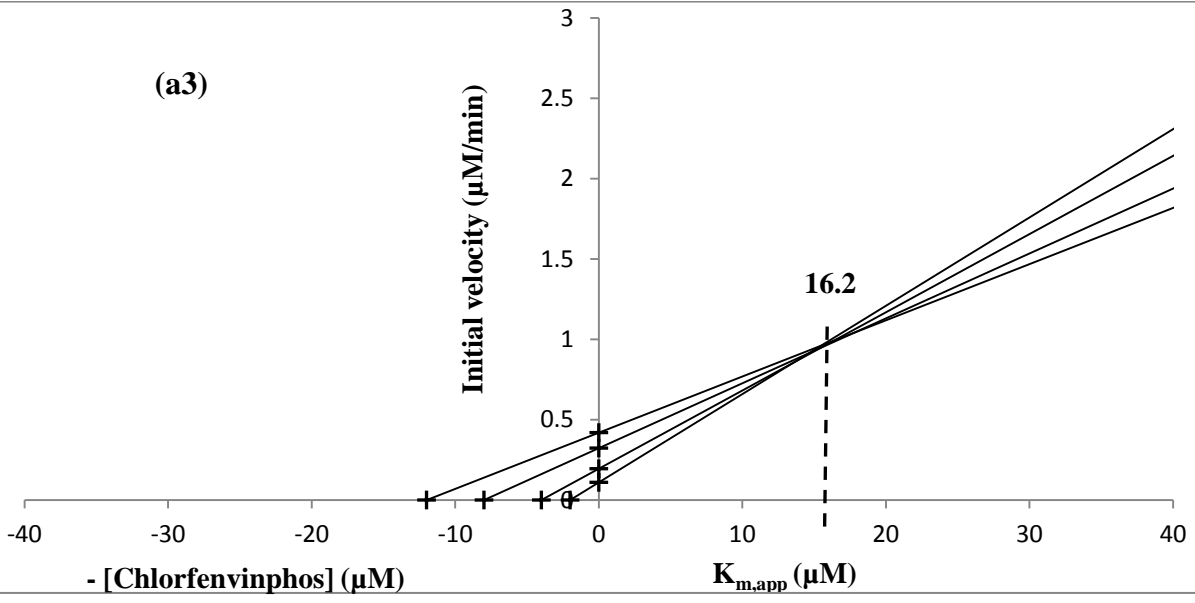
Fig. 2. Estimation of $K_{m,app}$ by the direct linear plot for: (a) chlorfenvinphos, (b) ethion and (c) linuron exposed to microsomes



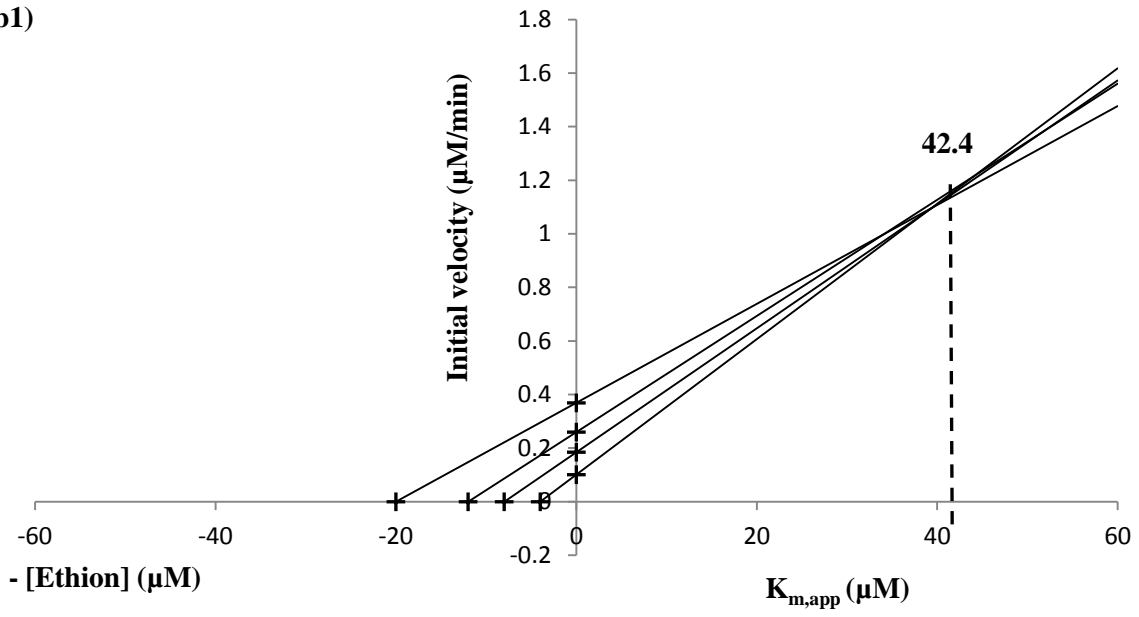
(a2)



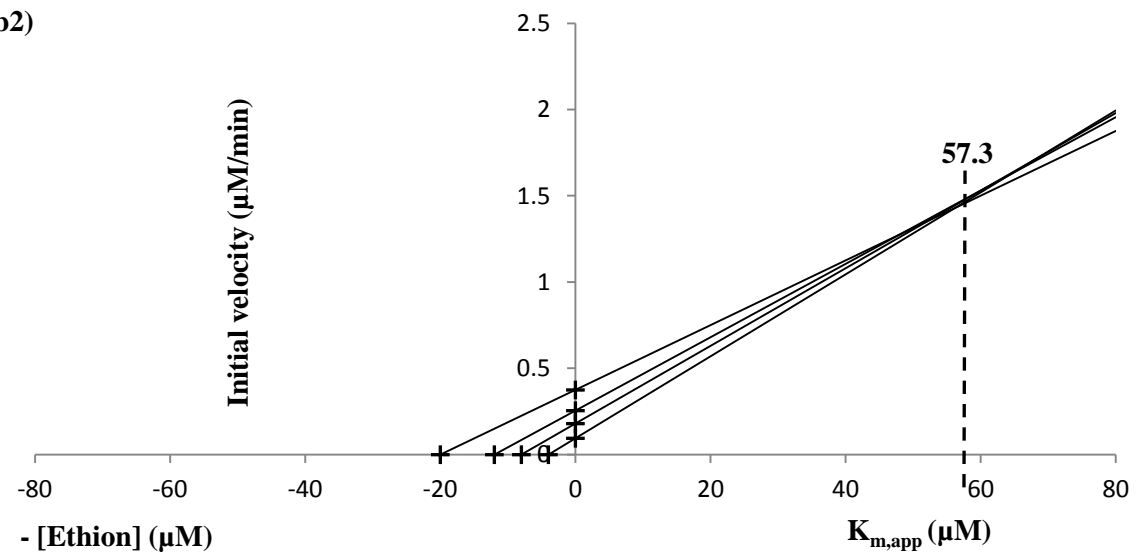
(a3)

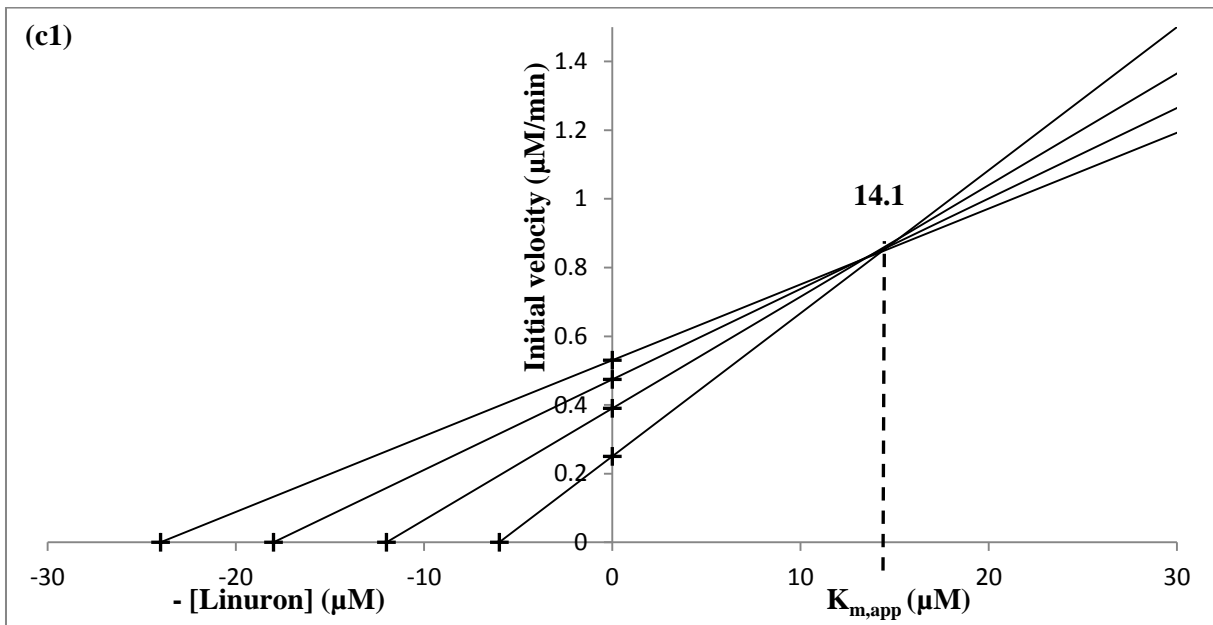
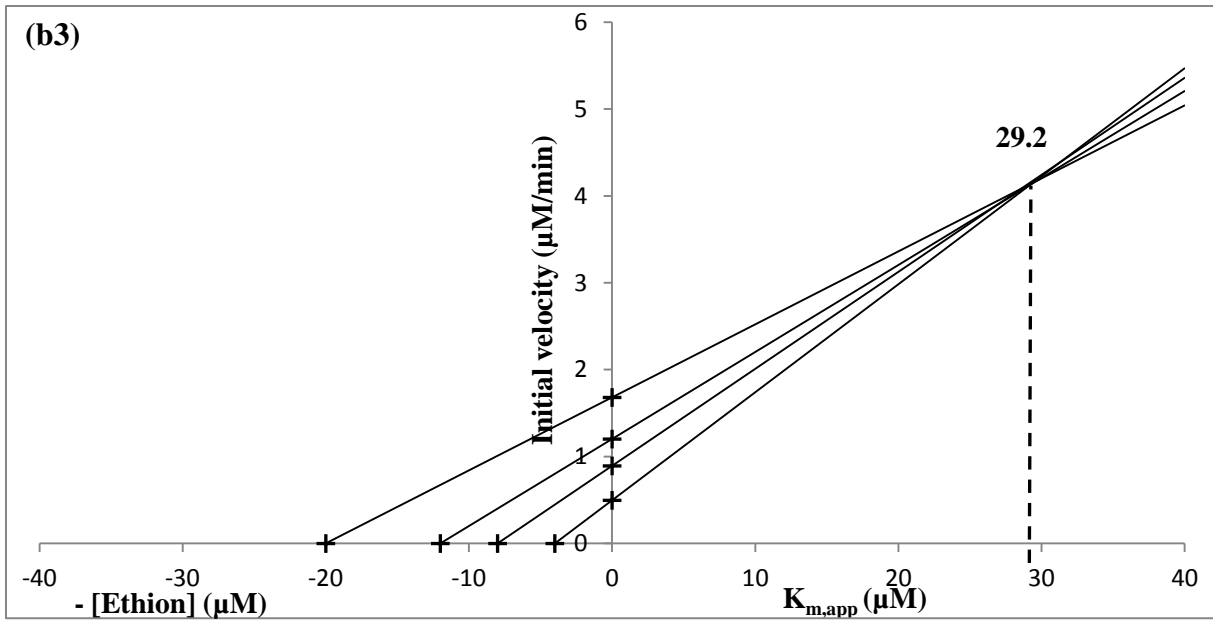


(b1)



(b2)





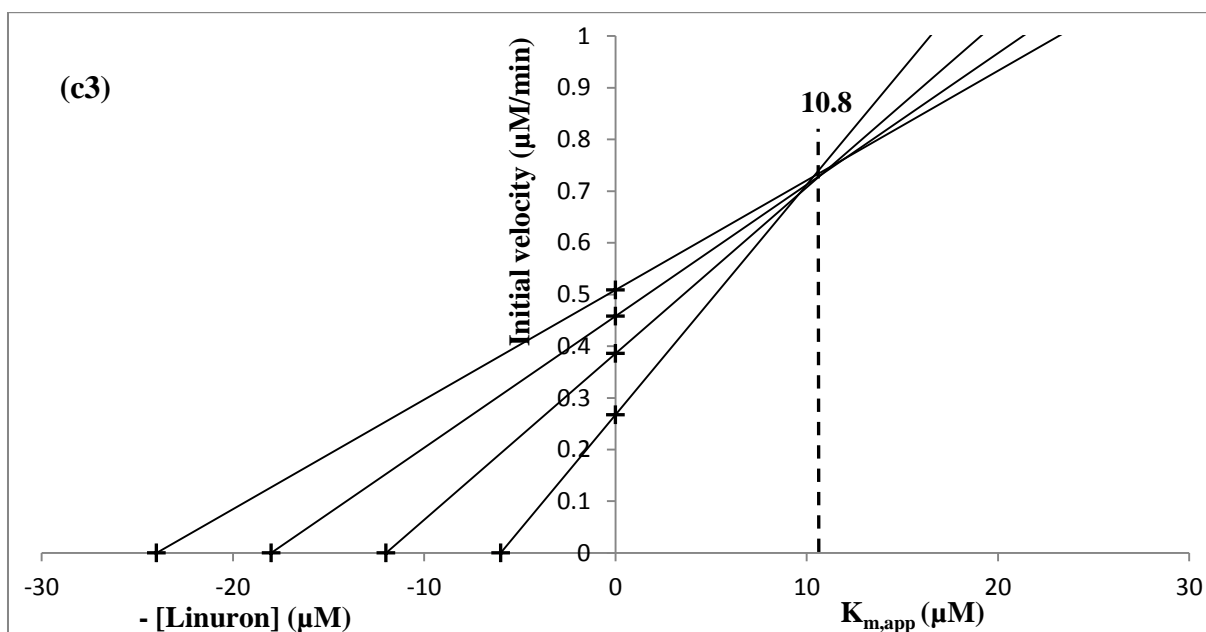
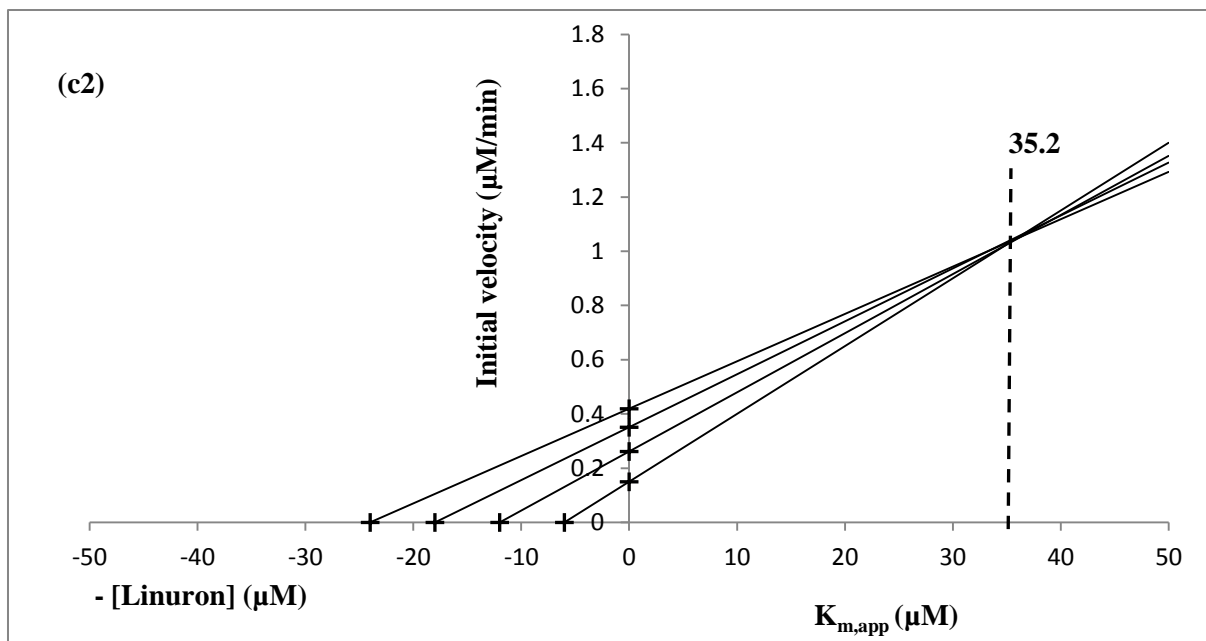


Fig. 3. Estimation of $K_{m,app}$ by the direct linear plot for human hepatocytes: (a1) chlorfenvinphos-M66, (a2) chlorfenvinphos-F31, (a3) chlorfenvinphos-F82, (b1) ethion-M66, (b2) ethion-F31, (b3) ethion-F82, (c1) linuron-M66, (c2) linuron-F31, (c3) linuron-F82.

ABSTRACT

General population exposure to pesticides mainly occurs via food and water consumption. However, their risk assessment for regulatory purposes does not currently consider the actual co-exposure to multiple substances. To address this concern, relevant experimental studies are needed to fill the lack of data concerning effects of mixture on human health. For the first time, the present work evaluated on human microsomes and liver cells the combined metabolic effects of chlorfenvinphos, ethion and linuron, three pesticides usually found in vegetables of the European Union. Concentrations of these substances were measured during combined incubation experiments, thanks to a new analytical methodology previously developed. The collected data allowed for calculation and comparison of the intrinsic hepatic clearance of each pesticide from different combinations. Finally, the results showed clear inhibitory effects, depending on the association of the chemicals at stake. The major metabolic inhibitor observed was chlorfenvinphos. During co-incubation, it was able to decrease the intrinsic clearance of both linuron and ethion. These latter also showed a potential for metabolic inhibition mainly cytochrome P450-mediated in all cases. Here we demonstrated that human detoxification from a pesticide may be severely hampered in case of co-occurrence of other pesticides, as it is the case for drugs interactions, thus increasing the risk of adverse health effects. These results could contribute to improve the current challenging risk assessment of human and animal dietary to environmental chemical mixtures.