Toxicology Letters 312 (2019) 173–180

Contents lists available at ScienceDirect

Toxicology Letters

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

Metabolism of triflumuron in the human liver: Contribution of cytochrome P450 isoforms and esterases

Rim Timoumi[a,b], Franca M. Buratti[c,*], Salwa Abid-Essefi[a], Jean-Lou C.M. Dorne[d], Emanuela Testai[c]

[a] Laboratory for Research on Biologically Compatible Compounds, Faculty of Dental Medicine, University of Monastir, Rue Avicenne, 5019, Monastir, Tunisia
[b] Higher Institute of Biotechnology of Monastir, Avenue Taher Haddad 5000, Monastir, Tunisia
[c] Istituto Superiore di Sanità, Environment & Health Dept., Viale Regina Elena, 299, Roma, Italy
[d] EFSA (European Food Safety Authority), Via Carlo Magno, 1A, Parma, Italy

ARTICLE INFO

Keywords:
Triflumuron
Human biotransformation
In vitro
Metabolism
Kinetics
Cytochromes P450 isoforms
Esterases

ABSTRACT

Triflumuron (TFM) is a benzoylurea insecticide commonly used in Tunisian agriculture and around the world to control crop pests and flies as a promising alternative to conventional insecticides for its arthropod specificity and low toxicity. From the evidence available in animal models, it can be expected that the metabolism of TFM is catalyzed by cytochrome P450 (CYP) and esterases. However, no data are available on human metabolism of TFM with regards to phase I metabolism and CYP isoform specificity. Hence, this manuscript describes experimental investigations to underpin *in vitro* phase I TFM metabolism in human samples for the first time. TFM biotransformation by recombinant human CYPs was characterized, then human liver microsomes (HLM) and chemical specific inhibitors have been used to identify the relative contribution of CYPs and esterases. Our results showed that all CYP isoforms were able to metabolize TFM with different affinity and efficiency. The relative contribution based both on the kinetic parameters and the CYP hepatic content was 3A4 > > 2C9 > 2C8 > 2A6 > 1A2 > 2B6 > 2D6 > 2C19 > 2C18 > 1A1 at low TFM concentration, whilst at high TFM concentration it was 1A2 > > 2C9 = 3A4 = 2A6 > 2C19 > 2B6 = 2C8 > 2D6 > 1A1 > 2C18. Experiments with HLMs confirmed the involvement of the most relevant CYPs in the presence of specific chemical inhibitors with a catalytic efficiency (Cl*app*) lower by an order of magnitude compared with recombinant enzymes. Esterases were also relevant to the overall TFM kinetics and metabolism, with catalytic efficiency higher than that of CYPs. It is foreseen that such isoform-specific information in humans will further support *in silico* models for the refinement of the human risk assessment of single pesticides or mixtures.

1. Introduction

Benzoyl-phenyl ureas (BPUs) insecticides are considered to be of low toxicity and have been used for the management of the widespread resistance of insects against conventional chemical insecticides (such as organophosphates (OPTs), carbamates, and pyrethroids) which could also represent a threat for the environment and human health. BPUs are chitin synthesis inhibitor (CSI) or non-systemic Insect Growth Regulators (IGR) causing toxicity and death by interfering with chitin biosynthesis and cuticle sclerotization during the molting of insects (Merzendorfer, 2013). This mode of action is considered as highly selective for the target organisms, being chitin present in the cuticle of insects, but completely absent in vertebrates and higher plants. The presence of chitin in other organisms, such as in the shell of crustaceans, could impact on some specific non target organisms. Some effects on non-target organisms, such as honey bees and catfish have been reported at environmentally relevant concentrations (Fine et al., 2017; Marimuthu et al., 2013).

Given its estimated agricultural area at 10.07 million hectares (FAO, 2016), Tunisia has resorted to the use of pesticides for crop protection. Indeed, the Country imports on annual average 3600 tonnes of pesticides per year (GAIN, 2014) with more than 330 active substances in use, mainly in the agriculture sector (Tunisian Health Ministry, 2017).

Among the pesticides present on the Tunisian market, Triflumuron (TFM) 2-Chloro-N - [[[4- (trifluoromethoxy) phenyl] amino] carbonyl] benzamide (Fig. 1), a second generation BPU, has been used so far mainly to control *Tuta absoluta,* a tomato leaf miner. Tomato is the first horticultural crop in Tunisia with a production area of 25,000 ha and a

* Corresponding author at: Istituto Superiore di Sanità, Environment & Health Dept, Viale Regina Elena, 299, 00161, Roma, Italy.
E-mail address: franca.buratti@iss.it (F.M. Buratti).

https://doi.org/10.1016/j.toxlet.2019.05.009
Received 5 March 2019; Received in revised form 3 May 2019; Accepted 9 May 2019
Available online 11 May 2019

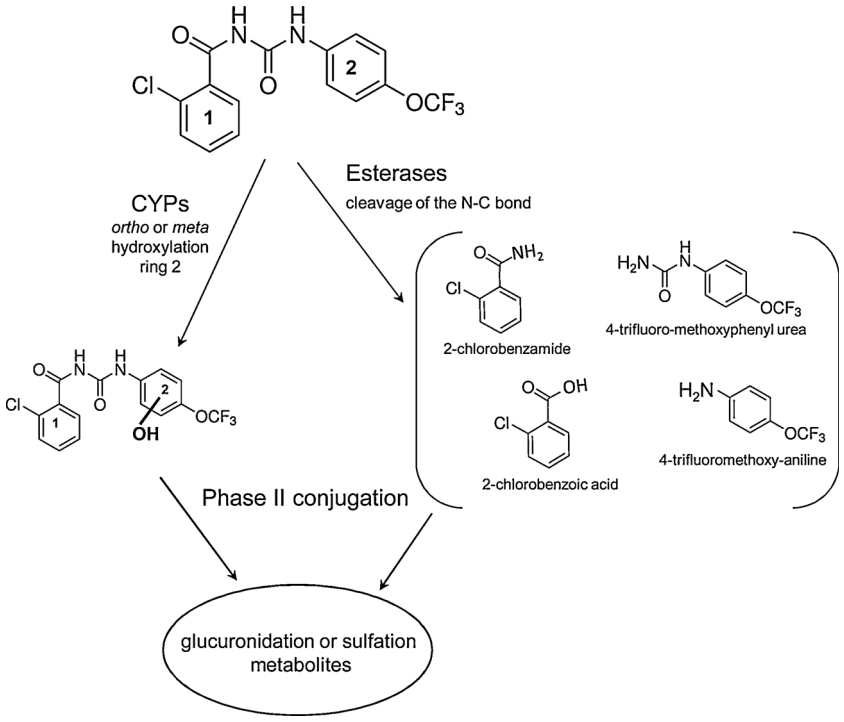


Fig. 1. Triflumuron chemical structure and expected metabolic pathway.

total harvest of 1.1 million metric tons (DGPA, 2009). To manage *T. absoluta*, a mixture of insecticides with different mode of action are used, such as spinosad, indoxacarb, pyrethroids and TFM because OPTs and carbamates are no more effective due to insect resistance. Furthermore, TFM is used also against locusts in the Sahel zone (Pan Africa, 2012).

At a global level, TFM and other BPUs are used on several fruits and vegetables, because of their relative low mammalian toxicity. It is registered in Europe (Commission Directive 2011/23/EU), in the USA and in Asia, where TFM residues have been reported in the Oolong tea infusing liquid (among the most favorite teas in China, Japan and Southeast Asia) with a level of 0.36 ng/ml (Chen et al., 2014), and in other countries in which significant pesticide residues were detected in crops (Safi et al., 2002; Nguyen et al., 2008) and in aquatic organisms (Olsvik et al., 2013). BPUs accounted for about 3.6% of the global insecticide market around the world in 2011 (Sun et al., 2015), and are also applied as biocidal products, with an anti-flea action for treating cats and dogs or to control parasites in aquaculture plants. Hence, exposure of the general population is widespread and not only related to residues in fruits and vegetables.

Beside its low acute toxicity in mammals, TFM shows erythrocyte damage as the main and critical toxic effect following repeated doses, independently on animal species, with induction of hemolytic anemia and secondary effects in spleen, liver and kidneys (EFSA, 2011). An increased methaemoglobin production was also reported in male Wistar rats treated orally with TFM for 28 days (Tasheva and Hristeva, 1993). The latter effect has been associated with the formation of the metabolite 4-trifluoro-methoxyaniline, which is present also as an impurity in the technical product, and characterized by an acute toxicity higher than the parent compound (EFSA, 2011). A clinical case of toxic methemoglobinemia was reported after deliberate ingestion of an unspecified BPU in one subject (D'sa et al., 2014). The hospitalized patient manifested cyanosis, low oxygen saturation and chocolate-brown colored blood, the methemoglobin concentration was 59.3%. Following treatment with ascorbic acid, there was evidence of haemolysis (D'sa et al., 2014). Therefore, although in a single case study, symptoms were confirmed in humans. Recently it has been reported that TFM *in vitro* using HepG2 cells was able to promote the metastasis of human liver cancer cells and that hypoxia-inducible factor (HIF)-1a is potentially responsible for these changes (Ning et al., 2018).

Limited information regarding the metabolic fate of TFM in mammals are available in the literature indicating hydrolysis, hydroxylation and then conjugation as the main metabolic pathways (with up to 26 components identified in bile) (EFSA, 2011). On this basis the involvement of esterases and cytochrome P450 isoforms (CYPs) can be expected as first step of metabolic pathway. However, data regarding TFM metabolism in human on an isoform- specific basis are not available. The involvement of CYPs was previously shown for other BPU insecticides (Ledirac et al., 2000; Sapone et al., 2005; Rudek et al., 2005; Abass et al., 2009; Elmadani et al., 2011).

In the context of current international risk assessment, the relevance of isoform-specific information is becoming increasingly important in characterizing the toxicokinetics of chemicals. A particular example is the Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances to be used in plant protection products, asks for specific kinetic parameters informing ADME, including an *in vitro* comparative metabolism study between pivotal species used in toxicity testing (e.g. rat, mouse, dog, rabbit) and human.

In the present work, we studied the contribution of the human liver microsomal CYPs and esterases in TFM metabolism, using an integrated approach, previously used for OPTs (Buratti et al., 2002, 2003, 2005), with single recombinant human CYPs and human liver microsomes (HLMs).

2. Material and methods

2.1. Chemicals

TFM (purity 98.8%), was purchased by Chem-Service (WestChester, PA). All other analytical grade chemicals were obtained from commercially available sources. Roche GmBh (Mannheim, Germany) supplied NADPH, NADP, NAD, glucose-6-phosphate (G6P) and G6P-dehydrogenase (G6PDH). The isoform-selective cytochrome P450 inhibitors

Table 1
CYPs marker activity in HLMs.

	1A2	2A6	2B6	2C8	2C9	2C19	2D6	3A4/5
HLM1	548	1416	726	2568	2532	77.4	259	3565
HLM2	680	1400	411	2930	2610	95.8	282	3390
HLM3	528	1310	513	2320	2130	106	230	2640

Results are expressed as pmol product/(mg prot · min)
Marker activity used by the supplier for HLM characterization: Phenacetin O-dealkylation (CYP1A2), Coumarin 7-hydroxylation (CYP2A6), Bupropion hydroxylation (CYP2B6), Amodiaquine N-dealkylation (CYP2C8), Diclofenac 4′-hydroxylation (CYP2C9), S-Mephenytoin 4′-hydroxylation (CYP2C19), Dextromethorphan O-demethylation (CYP2D6), Testosterone 6β-hydroxylation (CYP3A4/5).

Furafylline (1A2), Trolendamicyn (3A4), Orphenadrine (2B6) and Sulfaphenazole (2C family mainly 2C9) were supplied by Sigma-Aldrich (St. Louis, MO). Tetra isopropyl-pyrophosphoramide (iso-OMPA) from Sigma (St. Louis,MO) was used.

2.2. Biological samples: recombinant human CYPs and HLMs

cDNA-recombinant human CYPs, prepared from *Escherichia coli*-infected cells expressing single isoforms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2C18 and 3A4), were purchased from Xenotech Control Supersomes. The protein content was of 10 mg/ml and cytochrome P450 content was 1 nmol P450/ml, as indicated by the supplier.

HLMs from hepatic biopsies pooled from 200 donors (100 male and 100 female) (HLM1) and two 10 male donors containing different level of CYP (HLM2 and HLM3) were purchased from Xenotech. Protein concentration was 20 mg/ml; cytochrome P450 content was 0.411, 0.428 and 0.487 nmol/mg protein, respectively. HLMs were fully characterized by the supplier for the main monooxygenase activities by using selective model substrates for each single P450 (Table 1).

2.3. In vitro metabolism of TFM by recombinant human P450 isoforms and HLMs containing both CYPs and esterases

2.3.1. Enzymatic incubation

The standard incubation mixtures (0.5 ml final volume) contained 2 mM G6P, 2 mM MgCl2, 2–4 U/ml G6PDH, TFM (1–50 µM with single CYPs and 1–150 µM with HLM), 1 mM NADP, CYPs (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) (50 pmolP450/ml) or HLMs (1 mg protein/ml) in 50 mM Tris–HCl and 1 mM EDTA buffer (pH 7.4) (Buratti et al., 2005). The mixture was preincubated at 37 °C for 3 min before starting the reaction by adding 10 µl of NADP. The reaction was stopped after 2 h by adding 1.5 ml ice-cold ethylacetate. At least three independent incubations were carried out in triplicate. Different incubation times (30, 60, 120 and 240 min) were used in preliminary studies, in order to verify the linearity of the reactions *vs.* time (data not shown).

With HLMs, three different kind of experiments were carried out : i) when only the NADPH-generating system was present (standard incubation) the combined activity of esterases and CYPs was studied; ii) CYPs activity was measured by adding iso-OMPA 50 µM, an inhibitor of unspecific esterases, to the standard incubation (experimental conditions previously identified; Buratti et al., 2005); iii) by omitting both the NADPH-generating system or iso-OMPA, only esterases activity was measured.

In order to study the effect of the isoform-selective cytochrome P450 inhibitors with HLMs, 5 µM orphenadrine (ORF) or 20 µM furafylline (FUR) or 100 µM troleandomycin (TRO) or 25µM sulfaphenazole (SULF), as specific inhibitors of CYP2B6, 1A2, 3A4 and 2C family (mainly 2C9), respectively, were added in the iso-OMPA-containing standard mixture at two TFM concentrations (25 and 100 µM). A preincubation of 15 min was necessary for the three mechanism-based inhibitors ORF, FUR and TAO with the NADPH-generating system at 37 °C. Being SULF a competitive inhibitor, it was added concurrently with TFM, without pre-incubation.

Since the standards for TFM metabolites were not commercially available, the biotransformation was quantified by measuring the decrease of the parent compound. Consequently, controls were run in parallel with each sample and consisted of a standard incubation mixture with heat-inactivated biological specimen not endowed with CYPs and esterase activity.

2.3.2. Quantitative HPLC analysis of TFM metabolism

Once the incubation was terminated, with 1.5 ml ethylacetate, samples were vigorously shaken for 10 min and centrifuged at 3000 rpm for 10 min. The organic phase was collected, and the aqueous phase was re-extracted with 1.5 ml ethylacetate. The pooled organic phase was dried under nitrogen and re-suspended with 0.5 ml methanol, immediately before HPLC analysis. In preliminary study the extraction capacity of ethylacetate and dicloromethane was assessed. Ethylacetate was selected as the most efficient extraction solvent, since a single wash with ethylacetate already gave rise to a recovery > 90% also when the highest concentrations were used (data not shown).

The HPLC analysis were carried out with a Perkin Elmer Series 200 liquid chromatograph equipped with a Perkin Elmer diode array LC 235 detector and a Supelcosil LC18-DB (length = 25 cm; diameter = 4.6 mm) reversed phase column. The mobile phase (1 ml/min flow rate) consisted of (80:20, v/v) methanol:water both acidified with trifluoroaceticacid, TFA, (0.05%).

The retention time of TFM was 6.112 min. The injection volume was 20 µl. The absorption of the eluate was measured continuously at 240 nm. Using TFM concentrations range from 1 µM to 150 µM, a linear calibration curve with a correlation coefficient r = 0.9935 was obtained.

2.3.3. Data analyses

The kinetic parameters Km and Vmax have been obtained from linear or non-linear (Michaelis–Menten) regression fit curve with GraphPad 6 PrismTM software. The intrinsic clearance (CLi) was derived as the slope of the linear regression on the enzymatic activity curve or as the Vmax/Km ratio, for the non-linear activities. The unpaired *t* test was applied to determine the statistical significance ($p < 0.05$) of the inhibition data.

3. Results

3.1. Metabolism of TFM by single recombinant human CYPs

To study the biotransformation of TFM, CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6 and 3A4 were used with a range of concentrations of TFM (1–50 µM) by measuring the decrease of the parent compound.

In the range of TFM concentrations used all CYPs tested showed some activity, although with different behavior. Some CYPs, namely: 2C9, 2C8, 3A4, 2B6, 2A6, 2D6 and 2C18, showed a typical saturation curve (Fig. 2A, B), whereas others, such as 2C19, 1A2 and 1A1 remained linear also at the highest concentration tested (Fig. 2C).

The values of Vmax, Km and Cli obtained for each single recombinant CYP are reported in Table 2. CYP3A4 and two members of the 2C family (2C8 and 2C9) showed the highest affinity for TFM (meaning the lowest Km values), but were relatively rapidly saturated, having lower Vmax values with respect to other isoforms.

The CLi values range was 0.015 − 0.082 nmol product/(nmolP450·min·µM) with the following ranking 2C8 > 2B6 > 3A4 > 2C18 > 2D6 = 2A6 > 1A1 > 1A2 > 2C19.

The relative contribution (%) of each individual CYP to the total TFM metabolism was tentatively estimated, taking into account the average human hepatic content of each isoform, considering data

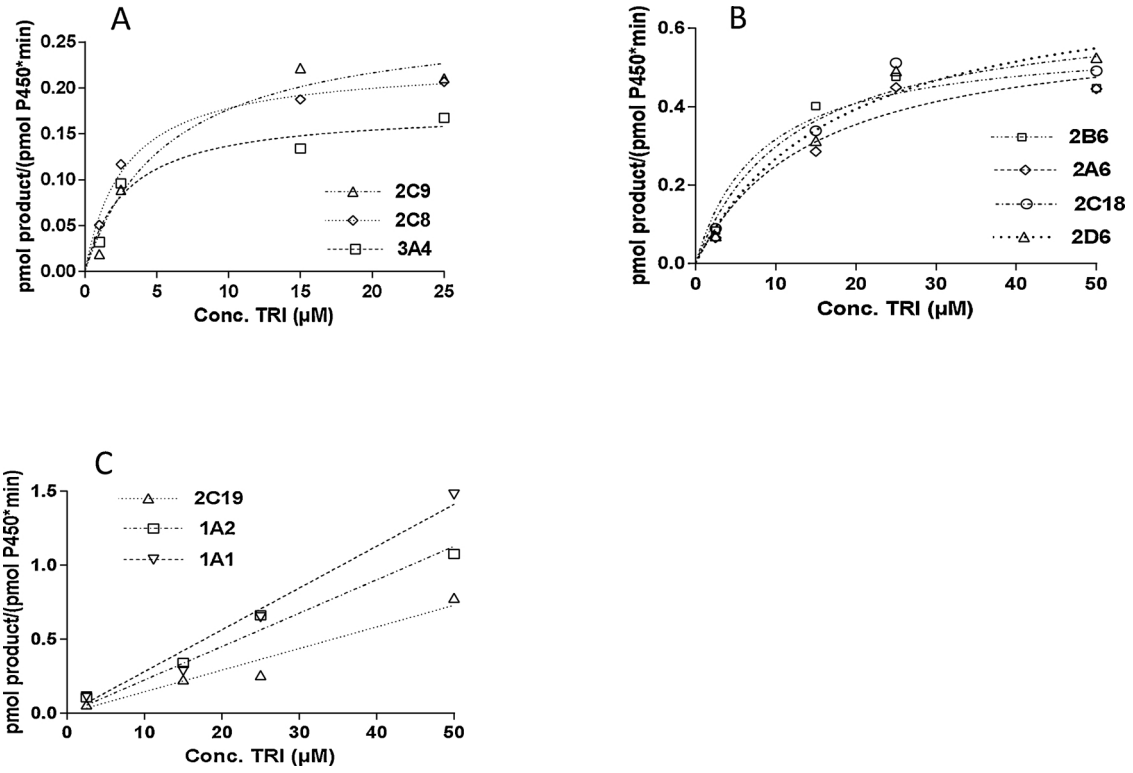


Fig. 2. Dependence of TFM metabolism catalysed by single recombinant human CYPs on substrate concentration.

Table 2
Kinetic parameters of TFM metabolism by single human recombinant CYPs.

	Vmax[a]	Km[b]	Cli[c]
2C8	0.227	2.8	0.081
2B6	0.574	8.1	0.071
3A4	0.176	2.9	0.061
2C18	0.654	11.9	0.055
2C9	0.279	5.8	0.048
2A6	0.615	14.7	0.042
2D6	0.746	17.9	0.042
1A1	–	–	0.028
1A2	–	–	0.023
2C19	–	–	0.015

[a] nmol product/(nmolP450 · min).
[b] μM.
[c] nmol product/(nmolP450 · min· μM).

reported in Pastrakuljic et al. (1997) and in Achour et al. (2014). The relative CYP contributions were calculated from CL*i* values of the active CYPs for low TFM concentration and from Vmax values for high TFM concentration, except for those CYPs showing linear activity, for which the rate detected at 50 μM was used (Fig. 3A and B).

Considering the CYP hepatic content, the ranking for the relative contribution toward TFM metabolism was different from the ranking based on *in vitro* CL*i* listed above and was also concentration-dependent. At low TFM concentration the major contribution was from 3A4 and 2C family, whilst at high TFM concentration CYP1A2 was the most relevant one (Fig. 3A and B).

3.2. Metabolism of TFM by HLMs

The tested HLMs were characterized by having most monooxygenase activities toward probe substrates at a similar level (Table 1). Once TFM metabolism was measured, the kinetics in HLM1 and HLM3 samples showed a linear behavior when the combined activity of CYPs and esterases was tested. In contrast, a typical saturation curve was

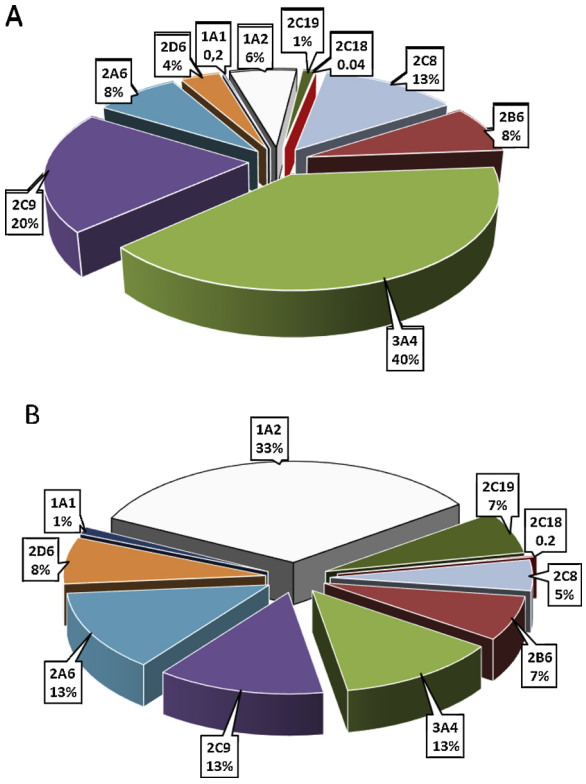


Fig. 3. Estimate of the relative contribution of CYP isoforms to TFM biotransformation, using kinetic parameters obtained with recombinant enzymes and the hepatic content (calculated by data from Pastrakuljic et al. (1997) and Archour et al. (2014)) at low (A) and high (B) TFM concentrations.

shown in the presence of the esterase inhibitor iso-OMPA, when only the CYPs were active in metabolizing TFM (Fig.4A and B). The saturation curve was characterised by kinetic parameters Km*app* and

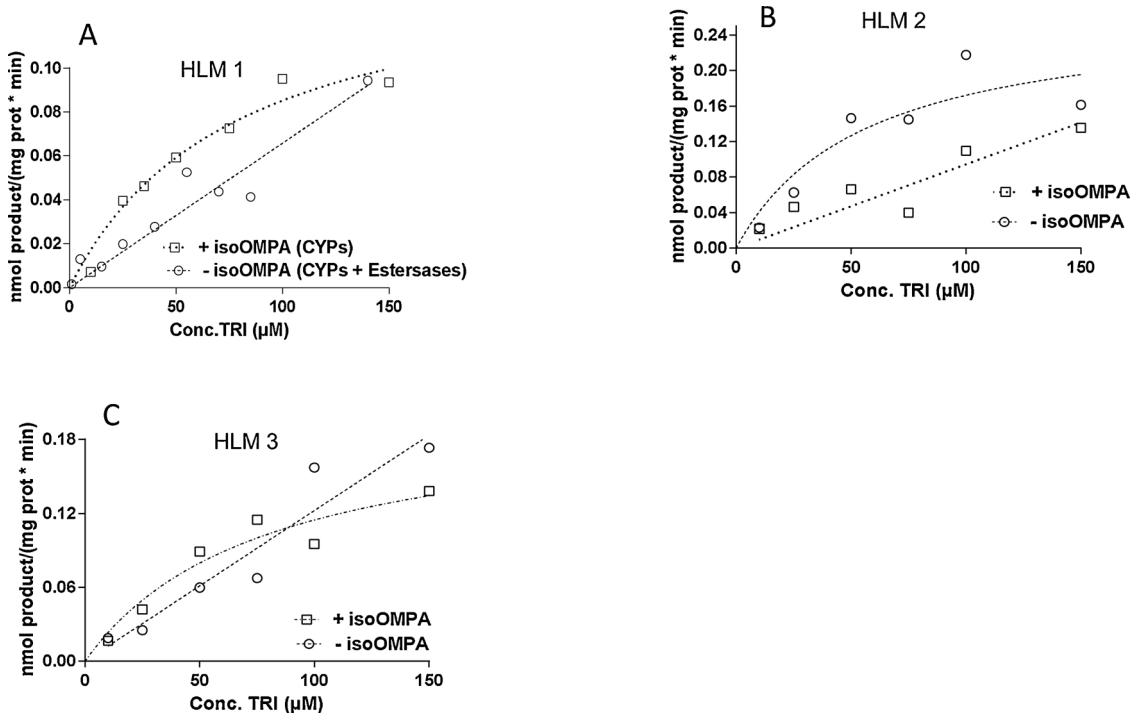


Fig. 4.) Dependence of TFM metabolism catalysed by HLMs on substrate concentration.

Vmax*app* lower by one order of magnitude, when compared with those calculated using recombinant enzymes.

To highlight the contribution of esterases in the metabolism of TFM, the NADPH-generating system have not been used with HLM1 and HLM3 and the kinetic showed a typical saturation curve (Fig. 5), with 2–4 fold higher affinity (lower Km*app*) and efficiency (higher Cli*app*) for TFM metabolism in comparison to CYP activity (Table 3). Kinetic parameters were very similar in the two samples. On the other hand, the CLi *app* for CYPs contribution alone or esterases alone was higher than the one measured for the combined activity esterases plus CYPs (Table 3). Although the results obtained with HLM1 and HLM3 were quite similar, the described kinetic behavior cannot be generalized, since a third HLM sample (HLM2) having a similar panel of CYP activities (Table 1) provided different results (Fig. 4C). This may be due to a variable esterase content, which unfortunately could not be checked due to the unavailability of additional amount of sample from the same batch.

To characterise the relative contribution of some CYP isoforms (1A2, 2B6, 2C19 and 3A4) in TFM metabolism, specific chemical inhibitors were used in the mixture (FUR (1A2), TRO (3A4), ORP (2B6) and SULF (2C family mainly 2C9) at two TFM concentrations (25 and 100 µM) (Fig. 6). The results confirmed the involvement of the 1A2, 2C19 and 3A4 for TFM metabolism without marked differences between low and high TFM concentrations (Fig. 6A and B): inhibition of the activity of different isoform ranged between 20 and 60% (the higher value being related to 2C family).

When all inhibitors were concurrently present a residual activity (on average about 30%–40%) remained, confirming the contribution of the other isoforms.

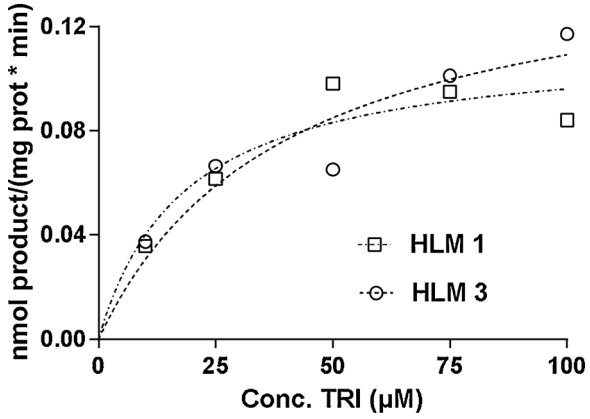


Fig. 5. Dependence on TFM concentration of metabolite formation catalysed by aspecific esterases in two different HLMs.

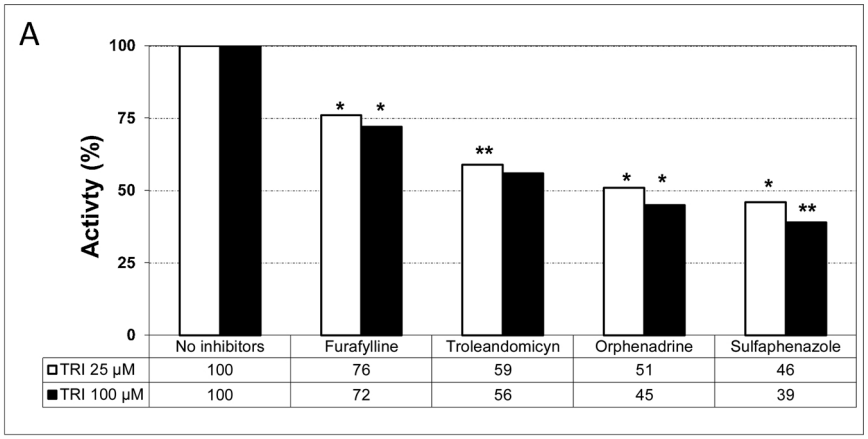
Table 3
Kinetic parameters of TFM metabolism by CYPs and/or esterases in HLMs.

	Vmaxapp[a]	Kmapp[b]	Cliapp[c]
CYPs + esterases			
HLM1			0.007
HLM2	0.2684	55.95	0.0048
HLM3			0.0012
CYPs			
HLM1	0.1515	78.2	0.0019 (0.0046)[d]
HLM2	–	–	0.0009 (0.0021)[d]
HLM3	0.2038	77.94	0.0026 (0.0053)[d]
Esterases			
HLM1	0.1138	18.42	0.0062
HLM3	0.1526	39.85	0.0038

[a] nmol product/(mg prot·min).
[b] µM.
[c] nmol product/(mg prot·min·µM).
[d] nmol product/(nmolP450·min·µM); CYPs content : HLM1 = 0.411 nmolP450/mg prot, HLM2 = 0.428 nmolP450/mg prot, HLM3 = 0.487 nmolP450/mg prot.

4. Discussion

In the human health area a global picture of TFM toxicokinetic and toxicological profiles is needed for quantitative risk assessment, as well



HML3

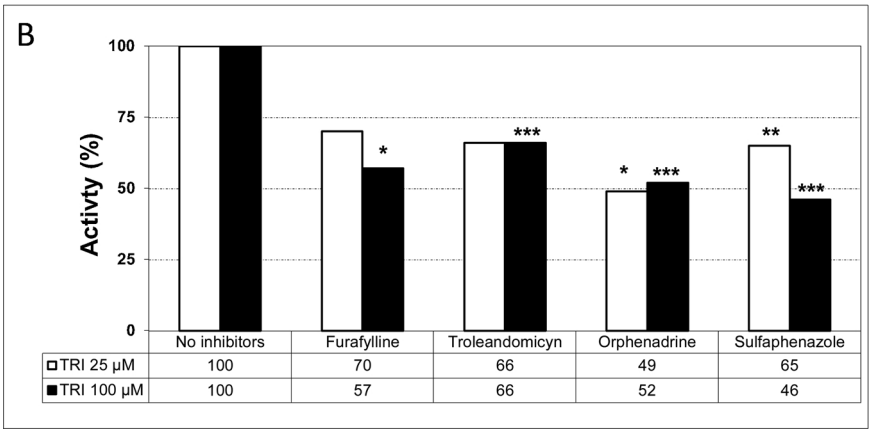


Fig. 6. Inhibition of TFM metabolism by some isoform-specific CYP inhibitors with two different HLMs (TFM concentration used: 25 and 100 µM). Stars indicate the statistically significant difference between the control incubation (without the inhibitor, set as 100% activity) and the activity measured in the presence of different inhibitors. * = $p < 0.05$; **=$p < 0.01$; ***$p < 0.005$.

as to inform PBPK models with isoform-specific *in vitro* parameters (e.g. Vmax, Km and Cli). This can be especially relevant considering that methaemoglobinemia, the most relevant toxic effect associated to TFM in mammals, has been attributed to the formation of a metabolite (4-trifluoro-methoxyaniline) (EFSA, 2011). Here, human liver phase I metabolism of TFM has been investigated for the first time using recombinant human cytochrome P450 isoforms and HLMs to characterise the relative contribution of CYPs and esterases, identifying the isoform-specific affinities and activities. The variability in TFM metabolism quantification assessed via the disappearance of the parent compound, was averaged out with an increased number of replicates, as reported above.

The use of *in vitro* concentrations close to the actual human exposure levels is crucial to obtain data relevant to the *in vivo* situation. The range of concentrations to be tested was kept as low as possible, compatibly with the reliable detection of parent compound depletion, and considering information on the possible presence of TFM as residue in food commodities. Considering the ADI value as identified by EFSA (0.014 mg/kg body weight (bw) per day) as the 'extreme worst case' for exposure and the almost complete absorption by the gastro-intestinal tract (EFSA, 2011), the blood circulating concentration in an adult of 60 kg bw (and a total Volume of distribution of about 13 L) would correspond to 0.33 µM. Since *in vivo* hepatic concentrations are usually higher than circulating ones, TFM concentrations < 50 µM could be easily achieved in exposed individuals and therefore the *in vitro* concentrations used here may be considered in the same order of magnitude of the *in vivo* situation.

The expected metabolic pathways for phase I metabolism of TFM, considering its chemical structure, are the following: hydroxylation of the aromatic ring in the *ortho* and *meta* position with respect to the $–OCF_3$ substituent, cleavage of the N−C bond to form 2-chlorobenzamide, 2-chlorobenzoic acid and 4-trifluoromethoxy-phenyl urea, 4-trifluoro-methoxyaniline and subsequent phase II metabolism via conjugation reactions (Fig. 1). It can be therefore anticipated that the main phase I enzymes catalyzing the first steps of TFM biotransformation are CYPs and esterases.

The combined effects of the high affinity and Cl*i* obtained with recombinant CYPs with their relative hepatic content have shown that at low TFM concentrations the major isoform contributing to its metabolism was given by CYP3A4 and 2C family, accounting together for about 80% of the liver CYP biotransformation capacity. All the other isoforms were active, each of them contributing for less than 10%, including CYP2B6, characterized by a relatively high Cli, but with a low hepatic content (Fig. 3A). At high TFM concentration, the relative contribution of CYP3A4 dropped, those by the 2C family slightly decreased, whereas CYP1A2 activity became the most relevant, contributing alone for more than 1/3 of the total metabolism (Fig. 3B). This result could be expected, since kinetics of CYP2C19 and CYP1A2 remained linear and was not saturated in our experimental conditions, even at the highest concentration tested. In this contest the importance of CYP1A2 can increase even more, particularly in the case of acute intoxication or poisoning with very high TFM concentrations. The CYP1A2 contribution at higher concentrations is likely to be underestimated, since the rate measured at 50 µM rather than the Vmax value was used in the calculation. The

situation is similar for CYP1A1, which, however, is present only as trace in the liver, and therefore its relevance is expected to be scant in any situation.

The involvement of those CYPs found to be the most relevant to TFM metabolism was further confirmed by results obtained with HLM in the presence of specific chemical inhibitors.

With HLMs where the different isoforms are concomitantly present and compete with each other for the substrate, a lower catalytic efficiency was observed, when compared to the single recombinant isoforms. This was evidenced by an order of magnitude lower Cli*app* value in the three samples. None of the CYP isoforms has a marked prevalence, as evidenced by the linear Eadie-Hofstee plots (data not shown). HLM1 and HLM3 shows very similar values and behavior (also when data were referred to the actual CYP content instead of protein content): since HLM1 was obtained by pooling 200 individuals of both genders, it could be considered that this is a 'general' behavior, with most of the interindividual differences being averaged out. In order to have a prediction of microsomal Cl*i* estimates of each CYP, the relative abundance (RA) method was used (Stringer et al., 2009). With this method the microsomal Cl*i* contributions for TFM was based on the average hepatic CYPs content (Achour et al. (2014); Pastrakuljic et al., 1997). The predominant role for CYP3A4 was estimated as well as the involvement of members of the 2C family (2C8 and 2C9) (Table 4), confirming results obtained with recombinant enzymes. On the other hand, only a limited contribution of CYP1A2 was calculated (Table 4). Also the Relative Activity Factor (RAF) approach was used (Stringer et al., 2009), although limited to some CYPs (2C9, 3A4, 2A6, 2D6) for which the same probe substrate was used by the suppliers to measure the isoform-specific activity in recombinant CYPs and in HLM1. Again, a predominant role for CYP3A4 was predicted (Table 4), although members of the 2C family other than 2C9 and 1A2 could not be checked.

Our data are in line with results reported on other BPUs. Hexalumuron and chlorfluazuron produced *in vitro* the reversible inhibition of a single human CYP, namely CYP2C9 (Abass et al., 2009; Elmadani et al., 2011) with IC_{50} values of 6.0 and 7.5 μM, respectively, suggesting a relevant role for this isoform in their metabolism. Furthermore, an *in vitro* study on human metabolism on derivatives of BPUs, which seems to exert *in vitro* antitumor activity, evidenced the involvement of several CYP isoforms mainly CYP3A4 and CYP1A1, but also CYP2C8, CYP3A5/A7 and CYP2D6 (Rudek et al., 2005). CYP perturbation in hepatic and lung microsomes were reported after treatment *in vivo* with diflubenzuron in male and female mice, after three repeated doses, evidencing induction at the 752 mg/kg bw and inhibition at the 1075 mg/kg bw (Sapone et al., 2005), which are however extremely high treatment doses. Diflubenzuron was reported to strongly inhibit CYP1A1 expression in HepG2 cells by acting as an AhR antagonist (Ledirac et al., 2000).

In contrast to results from HLM1 and HLM3, results from HLM2 (obtained from 10 male donors) showed different kinetics, still linear at the highest concentration tested. Since the CYP isoforms activity panel is quite similar, this may reflect a different content of esterases, competing with CYPs for the substrate. And indeed, the involvement of esterases, whose catalytic efficiency in the tested samples was even higher than CYPs, seems to be of relevance in TFM metabolism. Carboxylesterases (CE), although widely distributed in mammalian tissue, showed the highest levels expressed in the liver and the gastrointestinal tract (the first tissue encountered by orally ingested residues) and the brain (Satoh and Hosokawa, 1998). Differently from the rat, where the CE activity in plasma is quite high, in humans the serum CE is apparently lacking (Williams et al., 1989). In the human liver, CE activity is found predominantly in the microsomal fraction (Hosokawa et al., 1990). Microsomal hepatic forms were cloned and characterized; single-nucleotide polymorphisms, with possible variation in their activity, have been described (Marsh et al., 2004).

Therefore, in order to identify the possible interindividual differences in the toxicity of TFM, it is important to know not only the human CYPs isoform-specific biotransformation, but also the relative contribution of other enzymes to the total metabolism. This is crucial to correctly inform models used to predict *in vivo* human kinetics. According to our results, it can be expected that interindividual variability in the esterase activity is likely to have an impact on the overall kinetics for the first steps of TFM metabolism.

Other conventional insecticides, such as organophosphates and carbamates highly toxic for mammals are bioactivated/detoxified by the same enzymes (Buratti et al., 2002, 2005; Buratti and Testai, 2005; Abass et al., 2009) and possible interference in their metabolism due to TFM exposure cannot be excluded. Consequently, considering the increased interest for the toxicity of multiple chemical exposure, and the need to have information on kinetics and mode of action to conduct the related risk assessment (EFSA, 2018), the knowledge about the metabolic fate of chemicals, including BPUs, even if with low mammalian toxicity, can be useful for the human risk assessment.

Such isoform-specific data integrated with *in vivo* TK data will be useful to carry out quantitative *in vitro* to *in vivo* extrapolation for TFM as well as other compounds (e.g pesticides, food and feed additives, contaminants).

This study is part of an EFSA funded project (GP/EFSA/SCER/2015/01) which aims to identify and model human variability in both toxicokinetics and toxicodyamics to produce variability distributions for PB-PK models. It is foreseen that such isoform-specific information in humans will i) further support the use of quantitative *in vitro-in vivo* extrapolation models and physiologically-based kinetic models for human risk assessment of pesticides for single substances; ii) provide a basis to identify possible metabolic interactions between multiple substances concomitantly used and, to which the population can be co-exposed through generating isoform specific constant of inhibition values (Ki). The possible integration of this dataset with the variability distribution within the population for the involved enzymes could provide information about the interindividual variability of kinetics, providing indication for chemical specific assessment factors.

Table 4
RA, RAF and CLi of CYPs in HLM1.

CYPs	RA[a]	Cl_{iCYP}[b]	RAF[a]	Cl_{iCYP}[b]
2C8	25	0.0020		
2B6	16	0.0012		
3A4	99	0.0060	142	0.0087
2C9	66	0.0032	35	0.0017
2A6	29	0.0012	85	0.0036
2D6	12	0.0005	3.4	0.0001
1A1	1	0.00003		
1A2	41	0.0009		
2C19	12	0.0002		
2C18	0.4	0.00001		

RA (Relative Abundance) for a CYP is the ratio: (Total HLM P450 concentration/100) * A, where A is the percent content of the CYP in the HLMs (extrapolated from Achour et al. (2014) and Pastrakuljic et al. 1997)).
CliCYP related to TRI in HLM1 for each isoform was calculated as: TRI-ClirecCYP * RA). (Stringer et al., 2009)
RAF (Relative Activity Factor) is the ratio CliHLM/ClirecCYP obtained with the same probe substrate.
CliCYP related to TRI in HLM1 was calculated as: TRI-ClirecCYP * RAF). (Stringer et al., 2009)
[a] pmolCYP/mg prot.
[b] nmol product/(mg prot · min·μM) (microsomal CLint contributions for TFM from each CYPs).

Conflict of interest statement

No conflicts of interest exist regarding this study.

Disclaimer

The views in this publication do not necessarily represent those of EFSA and are the authors only.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Funding

This project was partially supported by :

- The 'Ministère Tunisien de l'Enseignement Superieur et de la Recherche Scientifique' (Laboratoire de Recherche sur les Substances Biologiquement Compatibles, LRSBC).
- the European Food Safety Authority (EFSA) under the grant agreement no. GA/EFSA/SCER/2015/01.

Transparency document

The Transparency document associated with this article can be found in the online version.

References

Abass, K., Turpeinen, M., Pelkonen, O., 2009. An evaluation of the cytochrome P450 inhibition potential of selected pesticides in human hepatic microsomes. J. Environ. Sci. Health B 44 (6), 553–563.

Achour, B., Barber, J., Rostami-Hodjegan, A., 2014. Expression of hepatic drug-metabolizing cytochrome P450 enzymes and their intercorrelations: a meta-analysis. Drug Metab. Dispos. 42, 1349–1356.

Buratti, F.M., Testai, E., 2005. Malathion detoxification by human hepatic carboxylesterases and its inhibition by isomalathion and other pesticides. J. Biochem. Mol. Toxicol. 19, 406–414.

Buratti, F.M., Volpe, M.T., Fabrizi, L., Meneguz, A., Vittozzi, L., Testai, E., 2002. Kinetic parameters of OPT pesticide desulfurationby c-DNA expressed human CYPs. Environ. Toxicol. Pharmacol. 11, 181–190.

Buratti, F.M., Volpe, M.T., Meneguz, A., Vittozzi, L., Testai, E., 2003. CYP-specific bioactivation of four organophosphorothionate pesticides by human liver microsomes. Toxicol. Appl. Pharmacol. 186, 143–154.

Buratti, F.M., D'Aniello, A., Volpe, M.T., Meneguz, A., Testai, E., 2005. Malathion bioactivation in the human liver: the contribution of different cytochrome P450 isoforms. Drug Metab. Dispos. 33, 295–302.

Chen, L., Chen, J., Guo, Y., Li, J., Yang, Y., Xu, L., Fu, F., 2014. Study on the simultaneous determination of seven benzoylurea pesticides in Oolong tea and their leaching characteristics during infusing process by HPLC-MS/MS. Food Chem. 143, 405–410.

D'sa, S.R., Victor, P., Jagannati, M., Sudarsan, T.I., Carey, R.A., Peter, J.V., 2014. Severe methemoglobinemia due to ingestion of toxicants. Clin. Toxicol. (Phila). 52 (8), 897–900.

DGPA, 2009. Direction Générale de la Production Agricole. Tunisian Ministry of Agriculture. Annual Report.

FSA (European Food Safety Authority) Journal, 2011. 1941 Conclusion on the Peer Review of the Pesticide Risk Assessment of the Active Substance Triflumuron. 9(1). .

EFSA (European Food Safety Authority) Journal, 2018. Draft Guidance on Harmonised Methodologies for Human Health, Animal Health and Ecological Risk Assessment of Combined Exposure to Multiple Chemicals. Available at:. https://www.efsa.europa.eu/sites/default/files/consultation/consultation/180626-1-ax1.pdf.

Elmadani, M.F., Moustafa, Y.M., El-Azab, M.F., Olavi Pelkonen, O., 2011. Investigation of mechanism based inhibition of CYP450 enzymes by selected organophosphorous, pyrethroids and benzoyl urea pesticides in pooled human liver microsomes. Therapeut. Pharmacol. Clin. Toxicol. 15 (3), 197–204.

FAO, 2016. Country Profiles: Tunisia. Available at:. http://www.fao.org/countryprofiles/index/en/?iso3=TUN.

Fine, J.D., Mullin, C.A., Frazier, M.T., Reynolds, R.D., 2017. Field residues and effects of the insect growth regulator novaluron and its major Co-Formulant N-Methyl-2-Pyrrolidone on honey bee reproduction and development. J. Econ. Entomol. 110 (5), 1993–2001.

GAIN (Global Agricultural Information Network) Report Number: TS1407, 2014. Tunisia Food and Agricultural Import Regulations and Standards - Narrative FAIRS Country Report. . http://agriexchange.apeda.gov.in/IR_Standards/Import_Regulation/FoodandAgriculturalImportRegulationsandStandardsNarrativeTunisiaTunisia9192014%202.pdf.

Hosokawa, M., Mak, iT., Satoh, T., 1990. Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. Arch. Biochem. Biophys. 277, 219–227.

Ledirac, N., Delescluse, C., Lesca, P., Piechocki, M.P., Hines, R.N., de Sousa, G., Pralavorio, M., Rahmani, R., 2000. Diflubenzuron, a benzoyl-urea insecticide, is a potent inhibitor of TCDD-induced CYP1A1 expression in HepG2 cells. Toxicol. Appl. Pharmacol. 164 (3), 273–279.

Marimuthu, K., Muthu, N., Xavier, R., Arockiaraj, J., Rahman, M.A., Subramaniam, S., 2013. Toxicity of buprofezin on the survival of embryo and larvae of African catfish, Clarias gariepinus (Bloch). PLoS One 8 (10), e75545.

Marsh, S., Xiao, M., Yu, J., Ahluwalia, R., Minton, M., Freimut, R.R., Kwok, P.Y., McLeod, H.L., 2004. Pharmacogenomic assessment of carboxylesterases 1 and 2. Genomics. 84, 661–668.

Merzendorfer, H., 2013. Chitin synthesis inhibitors: old molecules and new developments. Insect Sci. 20 (2), 121–138.

Nguyen, T.D., Han, E.M., Seo, M.S., Kim, S.R., Yun, M.Y., Lee, D.M., Lee, G.H., 2008. A multi-residue method for the determination of 203 pesticides in rice paddies using gas chromatography/mass spectrometry. Anal. Chim. Acta 619 (1), 67–74.

Ning, X., Wang, Y., Yan, W., Li, G., Sang, N., 2018. Chitin synthesis inhibitors promote liver cancer cell metastasis via interfering with hypoxia-inducible factor 1α. Chemosphere 206, 231–237.

Olsvik, P.A., Samuelsen, O.B., Erdal, A., Holmelid, B., Lunestad, B.T., 2013. Toxicological assessment of the anti-salmon lice drug diflubenzuron on Atlantic cod Gadus morhua. Dis. Aquat. Org. 105 (1), 27–43.

Pan Africa, 2012. Briefing on Locust N° 3.

Pastrakuljic, A., Tang, B.K., Roberts, E.A., Kalow, W., 1997. Distinction of CYP1A1 and CYP1A2 activity by selective inhibition using fluvoxamine and isosafrole. Biochem. Pharmacol. 53 (4), 531–538.

Rudek, M.A., Zhao, M., Smith, N.F., Robey, R.W., He, P., Hallur, G., Khan, S., Hidalgo, M., Jimeno, A., Colevas, A.D., Messersmith, W.A., Wolff, A.C., Baker, S.D., 2005. In vitro and in vivo clinical pharmacology of dimethyl benzoylphenylurea, a novel oral tubulin-interactive agent. Clin. Cancer Res. 11, 8503–8511.

Safi, J.M., Abou-Foul, N.S., El-Nahhal, Y.Z., El-Sebae, A.H., 2002. Monitoring of pesticide residues on cucumber, tomatoes and strawberries in Gaza governorates, Palestine. Mol. Nutr. Food Res. 46, 34–39.

Sapone, A., Pozzetti, L., Canistro, D., Broccoli, M., Bronzetti, G., Potenza, G., Affatato, A., Biagi, G.L., Cantelli-Forti, G., Paolini, M., 2005. CYP superfamily perturbation by diflubenzuron or acephate in different tissues of CD1 mice. Food Chem. Toxicol. 43, 173–183.

Satoh, T., Hosokawa, M., 1998. The mammalian carboxylesterase. Annu. Rev. Pharmacol. Toxicol. 38, 257–288.

Stringer, R.A., Strain-Damerell, C., Nicklin, P., Houston, J.B., 2009. Evaluation of recombinant cytochrome P450 enzymes as an in vitro system for metabolic clearance predictions. Drug Metab. Dispos. 37, 1025–1034.

Sun, R., Liu, C., Zhang, H., Wang, Q.J., 2015. Benzoylurea chitin synthesis inhibitors. J. Agric. Food Chem. 63 (31), 6847e6865.

Tasheva, M., Hristeva, V., 1993. Comparative study on the effects of five benzoylphenylurea insecticides on haematological parameters in rats. J. Appl. Toxicol. 13, 67–68.

Tunisian Health Ministry, 2017. List of Insecticides and Rodenticide Controlled by the Ministry of Health. http://www.santetunisie.rns.tn/images/articles/liste-pesticidesavril2017.pdf.

Williams, F.M., Mutch, E.M., Nicholson, E., Wynne, E., Wright, P., Lambert, D., Rawlins, M.D., 1989. Human liver and plasma esterase. J. Pharma. Pharmacol. 41, 407–409.