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Relationship between exposure, body burden and target tissue concentration after oral administration of a low-dose mixture of pyrethroid insecticides in young adult rats

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ABSTRACT

Pyrethroids (PYRs) are synthetic insecticides increasingly used in agricultural and household pest control. Little is known on how the toxicity of highly effective bolus doses of single compounds compares to more realistic scenarios of low-level exposure to PYR mixtures. In this study, we examined a quaternary mixture of two noncyano (tefluthrin, TEF; bifenthrin, BIF) and two cyano (α-cypermethrin, α-CPM; deltamethrin, DTM) PYRs in young adult rats. These compounds are mostly composed of PYR isomers ranking top ten in acute lethality in rats. Concurrently, we administered near-threshold levels of the four PYRs dissolved in corn oil by oral route. Six hours later blood was collected and the liver and cerebellum were dissected out to determine PYR concentrations in these tissues using Gas Chromatography with Electron Capture Detector (GC-ECD). The mixture caused mild-to-moderate changes in non-locomotor behaviors and subcutaneous body temperature (up to +1.2–1.5 °C increase at 2–4 h after dosing, respectively, compared to pre-dosing records). The most toxic PYRs BIF and TEF reached higher concentrations in the cerebellum than the cyano-compounds α-CPM and DTM. In addition, PYR concentrations in the cerebellum were correlated to single compound proportions in the dosing solution and changes in body temperature. Our results suggest that aggregate exposures resulting in a target tissue burden of $\sim 10^{-1}$ nmoles PYR/g may be toxicologically relevant, expanding the evidence on exposure-dose-effect relationships for PYRs, and serving to design convenient pharmacokinetic models for environmentally relevant exposures to PYR mixtures.

1. Introduction

Pyrethroids (PYRs) are synthetic structural derivatives of a series of natural compounds with insecticidal activity named pyrethrins (Casida, 1980; Elliott, 1976). Most PYRs have been classified as Type I and Type II according to their chemical structure and acute neurotoxic effects in small rodents. Type I compounds lack an α-cyano group on the phenoxybenzyl moiety, and cause intense tremors (T-syndrome) in rats. Type II compounds contain an α-cyano group on the alcohol moiety, and cause repetitive bursts of pawing and burrowing, crawling, choreoathetosis, and profuse salivation as the dose administered increases (CS-syndrome). There are a few PYRs that produce mixed signs, including tremors and salivation, and have been accordingly classified as Type I/II (Soderlund et al., 2002; Wolansky and Harrill, 2008). Type I, type II and mixed-type PYRs have been long proposed to share a common primary mode of neurotoxic action. PYRs prolong inward sodium currents at voltage-gated sodium channels (VGSC) in targeted neurons. Thus, in conscious animals acute exposure to PYRs may induce prolonged nervous system hyperexcitation leading to neurophysiological collapse (Narahashi, 2000; Soderlund, 2012). Extrapolation from high dose toxicokinetics and effects of single PYRs in experimental animals to more realistic low-level exposure scenarios in humans requires the consideration of several influential factors. The canonical type I/II classification is mostly based on studies using single high bolus

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doses administered by intravascular (iv) or intracerebral (ic) routes in mice and rats, with clinical syndromes fully evolving through < 1 h after dosing (Lawrence and Casida, 1982; Verschoyle and Aldridge, 1980). A more diverse repertoire of neurobehavioral signs may appear along a few hours after oral exposure to PYRs in young adult rats (Wolansky and Harrill, 2008). Mild exacerbation of both motor activity and stereotyped behavior is observed soon after oral administration of middle-to-high effective doses, followed by dose-dependent decreases in activity later, regardless of the compound structure (Crofton and Reiter, 1988, 1984; Wolansky and Harrill, 2008). Moreover, low-effective exposure to PYRs causes mild increase in the core body temperature during the initial 30–90 min after oral dosing, regardless of the type, although compound-specific dose-related alterations in this endpoint (intense hyper- and hypothermia caused by Type I and Type II PYRs in adult rats, respectively) are observed after high-effective exposure at 120–180 min (McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky and Harrill, 2008; Wolansky et al., in preparation). PYRs may certainly have different actions and threshold levels in rats depending on the exposure conditions and the neurobehavioral endpoint (Wolansky and Harrill, 2008; Wolansky and Tornero-Velez, 2013). In humans, PYRs enter the body mostly via the oral route (pesticide residues in food; hand-to-mouth behavior in young children), and through the inhalation of environmental residues after the household pest control application of products containing PYRs as active ingredients (ATSDR, 2003; Julien et al., 2008; Li et al., 2014; Morgan, 2012; Tulve et al., 2006). Moreover, it is worth mentioning that environmental and human studies indicate that different patterns of combined exposure to PYRs may occur in general population (Haines et al., 2017; Morgan, 2012; Soderlund, 2012; Tornero-Velez et al., 2012b). A comprehensive understanding of health risks through the exposure to relevant mixtures of PYRs may thus require the assessment of different dosing and testing conditions (Wolansky and Tornero-Velez, 2013).

There are some gaps in the information about the existing relationship between PYR sample composition, absorption and distribution to target tissues, and dosage-related variations in the observed toxicity in rats and mice. In adult rats, brain concentration at ~6-9 h after oral exposure to the noncyano PYR bifenthrin (BIF) directly correlate with the severity of BIF actions in motor activity observed a few hours earlier (Scollon et al., 2011; Wolansky et al., 2007b). The same laboratory further examined the relationship between the dose administered, the tissue level (i.e., blood, liver, fat, and brain) and the motor activity alteration after acute oral joint exposure to low-effective doses of five PYRs (Hughes et al., 2016a; Starr et al., 2014, 2012). The test mixture in these studies consisted of a mix of isomer-rich compounds (deltamethrin [DTM] and esfenvalerate, both mostly consisting of 1 isomer; and β-cyfluthrin, featuring 2 out of 8 possible isomers), and racemic samples (cypermethrin [CPM] and permethrin, consisting of eight and four isomers, respectively). The brain was the tissue where individual PYR concentrations correlated best with single-compound ratios in the mixture dosing solution. Various toxicokinetic (TK) factors such as absorption rates, intestinal metabolism and decomposition mechanisms, and hepatic and blood binding proteins were proposed to contribute to PYR structure- and isomer-specific tissue disposition findings. Hence, a question worth asking is to what extent the mixture composition of the dosing solution and the testing endpoint may influence the relationship between PYR disposition into tissues and neurotoxicity. In this work, we evaluated a low-dose mixture of two CS-syndrome and two T-syndrome compounds in young adult rats to characterize the relationship between the administered dose, the target tissue dose and the effects of PYRs using subcutaneous body temperature as an endpoint.

2. Materials and methods

2.1. Animals

Hsd:WI Wistar rats (Animal Colony, Universidad de Buenos Aires; FCEN-UBA) were obtained at 8–9 weeks of age. As soon as they were received, all animals were housed two per cage in polycarbonate cages (45 cm × 24 cm × 20 cm) containing heat sterilized pine shavings, controlling for body weight balance between cages. All animals were maintained in the colony rooms on a 12:12 h photoperiod (0600:1800) at 22.5 ± 2.5 °C. Feed and tap water were provided ad libitum except when indicated. Experimental protocols were approved by UBA School of Science, Hygiene and Safety Department. Procedures recommended by NRC's Guide for the Care and Use of Laboratory Animals (8th edition) and FCEN-UBA Animal Colony Direction were followed to ensure reducing animal suffering to the least possible.

2.2. Chemicals

Test chemical samples were analytical grade (≥99% purity) except TEF (96.3% purity). BIF (CASRN 82657-04-3), 2-methyl-1,1-biphenyl-3-yl-methyl-(Z)-(1R)-cis-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyclopropane-carboxylate, consisted of 99% +(Z)-(1R)-cis isomer. DTM (CASRN 2918-63-5), (*S*)-α-cyano-3-phenoxybenzyl-(1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane-carboxylate, consisted of 98% +(*S*)-(1R)-cis isomer). α-CPM (CASRN 67375-30-8), (RS)-α-cyano-3-phenoxybenzyl-(1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate, is reported to be made up of +90% 1R and 1S configuration of the most active enantiomeric pair of the cis isomers of CPM (Pronk et al., 1996). BIF, DTM and α-CPM were purchased from ChemService (West Chester, PA, USA). TEF (CASRN 79538-32-2), 2,3,5,6-tetrafluoro-4-methylbenzyl-(Z)-(1RS)-cis-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2- dimethylcyclopropanecarboxylate, comprising equal amounts of the enantiomeric pair of +(Z)-(1R)-cis isomers (Knaak et al., 2012), was generously provided by Syngenta Argentina. The chemical structure and isomer composition of these PYRs are presented in Fig. 1. Chlorpyrifos (CASRN 2921-88-2), *O*,*O*-diethyl-O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate, used as an internal standard in the gas chromatographic determination of PYRs, was purchased from ChemService (West Chester, PA, USA). All organic solvents were of pesticide grade quality (Aberkon Química, Buenos Aires, Argentina).

2.3. Test mixture

The test mixture was intended to replicate a worst-case example of concurring exposure to pest control products formulated with highly toxic isomers of modern PYRs. Four criteria were used to select the number and identity of the compounds mixed up to prepare the test mixture. First, we selected four of the most toxic PYRs based on oral LD50 in adult rats (Wolansky and Harrill, 2008; WHO, 2010). Second, we considered the results of the First National Environmental Health Survey of Child Care Centers (CCC Survey; Tulve et al., 2006). This survey designated 334 child care buildings, from which 168 completed the survey. Tornero-Velez et al. (2012a,b) used a rigorous mathematical modeling and statistical analysis to characterize the distribution of PYR residues in the CCC study. These authors found two, three and four PYR compounds simultaneously occurring at 30, 15 and 10% of the CCC sites, respectively; co-occurrence of ≥5 PYRs at detectable levels was ≤ 2.5% of the total CCC sites sampled. Third, the detection frequency and maximum residue loading of DTM and CPM ranked top-ten among the PYRs analyzed in several environmental studies and food residue surveys (Jardim and Caldas, 2012; Morgan, 2012; Tulve et al., 2011, 2006). Last, we combined cyano and noncyano PYRs to blend the most common type-specific neurobehavioral syndromes that these insecticides may cause in rats (Wolansky and Harrill, 2008). Accordingly,

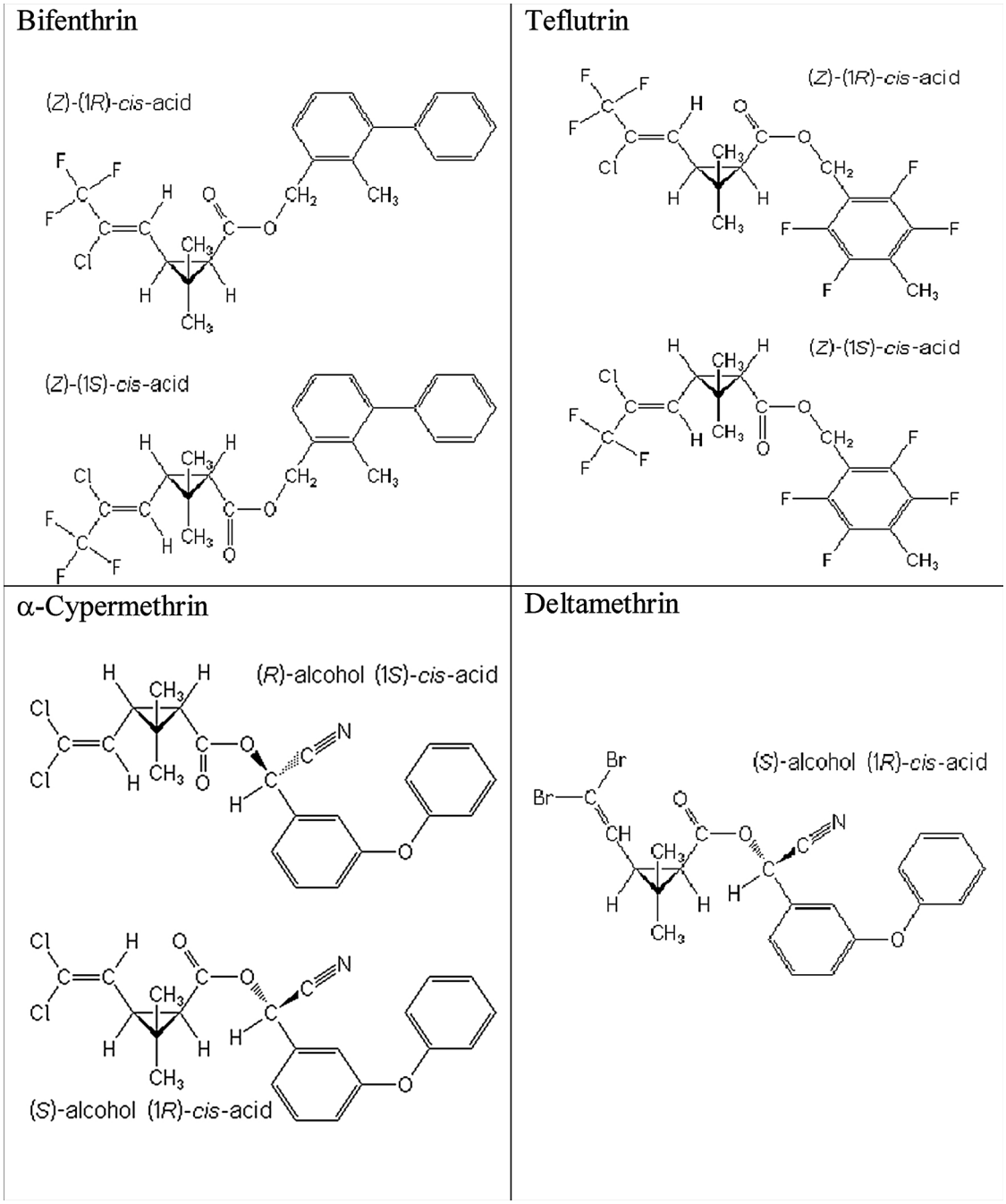


Fig. 1. Chemical structure of the PYRs examined.
Two noncyano (bifenthrin; tefluthrin) and two cyano (deltamethrin; α-cypermethrin) were used to prepare the test mixture. Chemical structures taken from http://www.alanwood.net.

in this study we were interested in examining a combination of two of the most toxic cyano-PYRs, DTM and α-CPM, plus the highly toxic noncyano-PYRs TEF and BIF.

We sought to combine low doses of the individual compounds well below threshold levels for PYR-specific signs of neurotoxicity such as salivation, whole body tremors and choreoathetosis based on previous neurobehavioral studies in adult rats (Mosquera-Ortega et al., 2013; Pato et al., 2011; Scollon et al., 2011; Wolansky et al., 2009, 2007a, 2007b, 2006). Moreover, we performed preliminary single-compound dose-response assays using identical dosing solution preparation protocol, vehicle and dosing procedures, and transponder-based technology to estimate low-effective benchmark dose (BMD) levels (i.e., BMD30s) for body temperature monitoring by BMDS modeling (Pato et al., 2011; Mosquera Ortega et al., in preparation). The absolute doses of each chemical in the stock solution (i.e., the highest mixture dose examined) were equal to 50% of the BMD30 for the chemical, which was a dose within or slightly off the 95% confidence intervals for threshold doses previously computed for motor activity (Wolansky et al., 2006). The time-dose-effect study included a vehicle control group and three PYR mixture doses (N = 4–6 per group): 8.950, 4.475 and 0.895 mg/kg bw (Table 1). Corn oil (Sigma-Aldrich Corporation, Saint Louis, MO, USA) was used to obtain diluted solutions of the stock mixture. All rats were ramdomly assigned to treatment groups.

Table 1
Composition of the stock mixture solution. The individual doses of the PYRs combined in the test mixture were near threshold levels based on pilot work, and previous studies using motor activity and core temperature as an endpoint (Wolansky et al., 2006, 2009; Wolansky et al., 2007a; Wolansky and Harrill, 2008). The total mixture doses examined were 8.950, 4.475 and 0.895 mg mixture/kg bw. Thus, each individual PYR dose level was *a priori* not expected to cause evident alterations in normothermia.

Compound	Low-effective level for Tsc, mg/kg	Single PYR dose, mg/kg	Mixing ratio, dose PYRi / total mixture dose	Threshold level [CI95%][a]	Expected contribution to the acute oral neurotoxicity
BIF	6.82	3.41	0.38	1.3 [0.7–1.9]	Near-threshold
TEF	5.22	2.61	0.29	0.9 [0.5–1.3]	Near-threshold
α-CPM	4.06	2.03	0.23	3–10[b]	Near-threshold[b]
DTM	1.80	0.90	0.10	1.0 [0.5–1.5]	Near-threshold
Total	17.90	8.95	1.00		

[a] Taken from Wolansky et al. (2006).
[b] Based on Pronk et al. (1996), and pilot work (data not shown).

2.4. Implant of transponders

Subcutaneous body temperature (Tsc) was monitored as an index measure of effect using microchip technology based on implantable subcutaneous transponders (Bio Medic Data Systems, Seaford, DE). Features of this transponder and its use in neurobehavioral studies have been already reported (Bardullas et al., 2015; Kort et al., 1998; Williams et al., 2007). Animals were transferred from the holding room to the adjacent testing room at least 48 h before transponder implant. Implant procedures were conducted at ~1 week before the test day. Briefly, antisepsis is carried out by cleansing the lowest portion of the interscapular region with ethanol 96%, and a sterile biocompatible glass-encapsulated thermistor (Implantable Programmable Temperature Transponder IPTT-300®; size: length, 14 mm; Ø = 2 mm) is then subcutaneously injected in the rat back using a pre-loaded sterile syringe. Rats were confirmed to recover using daily cage-side observation to ensure that no implant-related stress was affecting the susceptibility to PYRs on the test day.

2.5. Animal treatment

Fresh dosing solutions were prepared based on percentage of active ingredient in the sample 2–3 h before dosing by dissolving test chemicals in corn oil. Animals were deprived of water and feed one hour before dosing and through the entire temperature monitoring period. Dosing solutions were stirred and heated (40–50 °C) to ensure full solubility, and then administered at room temperature by oral route using a gavage procedure and round-tip stainless steel animal feeding needles (18 G, 2.0-in. long; Popper and Sons, New York, USA) connected to a 1-mL plastic syringe. Dose groups were balanced for body weight and dosing run order. Dosing was conducted by removing each animal from its cage and administering a bolus dose according to its body weight (range: 0.30–0.36 mL; dose volume rate = 1 mL/kg).

2.6. Body temperature monitoring system

Tsc signals emitted by subcutaneously implanted transponders were captured by a scanner-probe (model SP-6005) connected to a control unit (model DAS-6010 Mini Tower System), and transformed to °C units using the DAS-Host™ Windows-based application software (Bio Medic Data Systems; Seaford, USA). Temperature measures obtained using transponder-based systems or infrared thermometers have been reported to fairly correlate with core body temperature (Hershey et al., 2014; Vlach et al., 2000). Scans were conducted by sliding out cages from the cage rack, pulling off the cage cover, and placing the probe tip at a few mm distance from the animal's back implant point.

2.7. Mixture assessment

The time-dose-effect relationship for the simultaneous action of the PYRs in body temperature was obtained using previously reported procedures (Bardullas et al., 2015; Pato et al., 2011). Two blocks were required to complete the study. Vehicle-control animals were included in each block. Rats are primarily nocturnal animals. Accordingly, we made some ambient noise and gentle handling of all cages in the testing room to attenuate the impact of the different levels of physiological and behavioral activation on the Tsc measured in the experimental animals. This way, we also confirmed that the expected nonlocomotor and ambulatory behaviors were present in all animals at the beginning of the test day. Tsc scans were taken at 30 min prior to the dosing time to obtain pre-dose baseline for every animal starting at 9:30 AM. In each block, completion of dosing of all animals at zero time took ~25 min. Tsc monitoring and cage-side observations were then conducted at 30-min intervals for 4.5 h. Collection of all Tsc scans took ~10–20 min per testing time.

2.8. Tissue dissection

Following the Tsc monitoring period, animals were deeply anesthetized by exposure to CO_2/O_2, blood samples (2-mL aliquots) were obtained by cardiac puncture and collected in ice-cold glass vials preloaded with heparin, and the liver and cerebellum were dissected out. In addition to a convenient location within the skull allowing for a rapid and reliable dissection, and a satisfactory sample size for analytical procedures, the cerebellum has been proposed to be a key contributor to PYR neurotoxicity in adult rats (Gray and Rickard, 1982; Cremer and Seville, 1985; Song and Narahashi, 1996; Abdel-Rahman et al., 2001; Dayal et al., 2001).

2.9. Determination of PYR levels in target tissues

2.9.1. PYR extraction

PYR concentrations were determined in blood aliquots (2 mL) and in liver and cerebellum samples (250–300 mg). The procedure to extract the PYRs from target tissues was similar to that reported by Scollon et al. (2011), with a modification of the homogenization protocol. In our study, Na_2SO_4 (anh) and saline solution were added to obtain a finely crumbled tissue preparation, to favor the salting out and to ease the subsequent organic extraction rounds. The PYRs in the homogenate were initially extracted with 5 mL hexane:acetone (80:20). Samples were then vortexed for 1 min, additionally shaken in a rotator-mixer shaker CK-Tech model Multi RS-60 (Biosan Ltd, Riga, Latvia) for 15 min, and finally centrifuged at 7000 rpm for 15 min. The organic layer was collected and the extraction procedure was repeated twice. Then, 5 µL chlorpyrifos internal standard solution (100 ng/mL) was added to each organic extract. The pooled organic layers were evaporated under a N_2 stream, and reconstituted in 1 mL hexane. The solid phase cleanup was carried out using a Visiprep SPE vacuum manifold workstation (Supelco Inc., Bellefonte, PA, USA). Extracts were loaded on Sep-pak solid phase extraction (SPE) columns (500 mg silica; Waters

Inc., Milford, MA, USA) preconditioned with 5 mL hexane. Each extract was washed with 5 mL hexane, and PYRs were eluted with 5 mL hexane:EtOAc (94:6). The purified extract was finally evaporated in N_2 at room temperature and reconstituted in hexane (200 µL).

2.9.2. Chromatographic conditions

The extract was analyzed on a Hewlett Packard Gas Chromatograph 5890 Series II, coupled with electron capture detector Ni^{63} (GC-ECD) (Hewlett Packard, PA, USA), and equipped with HP 6890 Series Autosampler and dual split/splitless injector and double column. Chromatographic separation was achieved using a DB-5 capillary column (30.0 m × 0.32 mm ID × 0.25 µm film thickness) (Phenomenex, Torrance, USA), and a PAS-1701 capillary column (25.0 m × 0.32 mm ID × 0.25 µm film thickness) (Hewlett Packard, USA) to confirm the analysis of PYRs. The following temperature program was set up: 80 °C for 1 min, then increasing to 190 °C at 30 °C/min, and finally increasing to 280 °C with a rate of 3.6 °C/min, and maintaining this temperature for 20 min. The injector and detector were operated at 270 °C and 300 °C, respectively. The N_2 carrier gas linear velocity was 1.4 mL/min. The injection volume was 2 µL in the pulsed splitless mode.

2.9.3. Method validation

All PYRs were quantified using a calibration curve. This curve was prepared using pure standard preparations of PYRs (i.e., TEF, BIF, α-CPM and DTM), and Chlorpyrifos in hexane as the internal standard. Seven concentration points covering the 0.25–500 ng/mL range were required. The calibration assays were conducted under similar conditions for every tissue examined. Homogenized blood, liver and cerebellum samples of adult nontreated rats of the same sex, body weight range and strain from the same animal colony were spiked with 100 µL of three standard solutions of the mixture of 1, 50 and 500 ng/mL (N = 3 per concentration). The percentage recovery was estimated by comparing the mean area under the curve (AUC) at chromatogram peaks corresponding to TEF, BIF, α-CPM and DTM, and the internal standard. The acceptable range for recovery was set at 80–120%. For each PYR, the limits of detection (LOD) and quantitation (LOQ) were measured using 10 replicates of the lowest calculated concentration. LOD and LOQ were computed from the calibration curve using the criterion of 3xSD for LOD and 10xSD for LOQ (Miller and Miller, 2005). Data below the estimated LOQ were not used. Last, reproducibility was evaluated by computing % CV, using three different concentration levels of the mixture, each injected in quintuple, along three independent runs.

2.10. Data and statistical analyses

Data were first stratified by PYR compound, tissue, time, and mixture dose. Graphics, descriptive statistics and inferential analyses were carried out using Statistica v.13 (TIBCO Software Inc., Palo Alto, CA, USA) and SigmaPlot v.14 (Systat Software, Inc., San Jose, CA, USA). In time-dose-response studies, Tsc data were analyzed using general linear mixed-effects (GLM) models with differences in Tsc from baseline (i.e., baseline-normalized Tsc, $\Delta T_i = T_{ti} - T_{t0}$; t0 = 30 min before dosing) as the dependent variable, dose and time as fixed factors, and subjects as random factor. F tests were performed to build RM-ANOVA tables for the mixed-effect models. Based on previous studies (Hughes et al., 2016b; Starr et al., 2014, 2012, Wolansky et al., 2009, 2006) and two single compound time-dose-effect studies of BIF (Scollon et al., 2011) and CPM (McDaniel and Moser, 1993), peak target tissue concentrations of PYRs and the most intense manifestation of neurobehavioral signs were presumed to occur at 1.5–3.5 h after dosing. Moreover, laboratory rats may show a mild increase in core temperature sustained for 30–90 min as a physiological response to handling and the intubation (oral gavage) procedure (Gordon, 2005; Wanner et al., 2015). Hence, for some of the statistical analyses, the Tsc data obtained at 30–90 min were excluded from consideration. Correlation analysis was used to relate changes in Tsc to PYR concentration in tissues, and to examine the relationships between the dose administered, tissue concentration, global dose of PYRs in the cerebellum and effects. Furthermore, the proportions of individual PYRs in the target tissues and the mixture dosing solution were graphically compared using a hierarchical clustering analysis of similarity. In all cases, a default p-value of 0.05 was used to test the significance of the main effects and post-hoc contrasts.

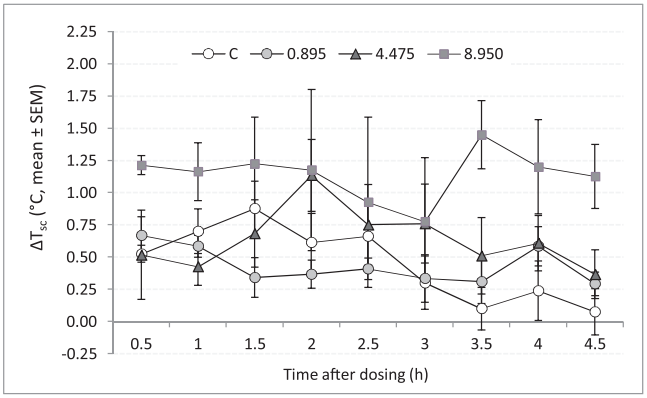
3. Results

We studied an acute toxicity schedule of exposure to an oral bolus dose of a quaternary mixture of PYRs. From oral dosing to sacrifice we observed no mortality. The two higher mixture doses caused mild-to-moderate signs of toxicity. Transient episodes of grooming, scratching, head and whole body shakes, and chewing appeared in most animals at higher doses. Chewing was also noticed at the lowest dose soon after dosing. Moreover, repetitive movements of the forelimbs and burrowing were occasionally evident. In general, these clinical signs started to be observed at 30–60 min after dosing, and extended for up to 1.5–2 h, returning to control-like behavior at ~3.5 h after dosing. No signs of high-dose PYR-specific toxicity such as excessive salivation, whole-body tremors, choreoathetosis, and aggression or else bizarre responses (McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky and Harrill, 2008) were observed.

There was no significant difference in the baseline Tsc between experimental groups. The pre-dose subcutaneous temperature ranged between 36.1 and 36.5 °C (global mean ± SD, 36.3 ± 0.5 °C). Fig. 2 (panel A) shows the baseline-normalized data for all the experimental groups along the entire Tsc monitoring period. The stock mixture caused alterations in Tsc. The time-course data revealed a trend for a mild-to-moderate increase in Tsc showing its peak at ~2–4 h after mixture dosing. While animals administered the intermediate dose had mostly recovered a control-like pattern of Tsc by the end of the monitoring period (Fig. 2A), effects after dosing the highest mixture dose were still evident at sacrifice. Taking the peak-effect period for analysis, the main effects of the dose and the dose*time interaction were not significant ($p > 0.10$), and there was a significant effect of time ($F_{4,63} = 2.99$; $p = 0.025$). Fig. 2B shows the responses of the animals administered with the highest mixture dose in three ways to allow for a more comprehensive interpretation of this figure. In this panel, the average normalized Tsc of vehicle-control animals at each monitoring time ($\Delta T_{sc\text{-}control}$) was substracted from the normalized responses of highest-dose group animals. Noteworthy, a clear difference was observed in a single animal compared with the rest of the highest-dose group. In this animal, a profound decline trend in Tsc was observed from 0.5 to 2.5 h after dosing, followed by an apparent recovery of the highest-dose group pattern at 4–4.5 h. This was an atypical response (denoted as "AR" in Fig. 2B) regarding the ΔTsc data obtained for the rest of the animals in this dose group. After excluding this AR response for the data set of this group, the RM-ANOVA showed significant effects of the dose ($F_{3,59} = 4{,}64$; $p < 0.021$) and time ($F_{4,59} = 4.25$, $p < 0.004$), but the interaction was not significant ($p < 0.05$). Pairwise comparisons by the Bonferroni *t*-test showed that the increase in normalized Tsc was significant in the highest-dose group vs. control, and vs. the lowest-dose group ($p < 0.05$). Thus, Fig. 2B shows that this AR animal had a major impact on the average normalized Tsc pattern of the highest-dose group depicted in Fig. 2A.

The no-effect level appeared at 0.895 mg/kg, and the activity of the intermediate mixture dose was assumed to be between a threshold and a low effective level (Fig. 2A). This nearly effective dose was consistent with the threshold dose computed in a first study of a mixture of 11 chemicals using similar dosing conditions and motor activity as an endpoint (Wolansky et al., 2009), and slightly below the threshold dose in a subsequent study of the same laboratory testing a 5-chemical

Panel A



Panel B

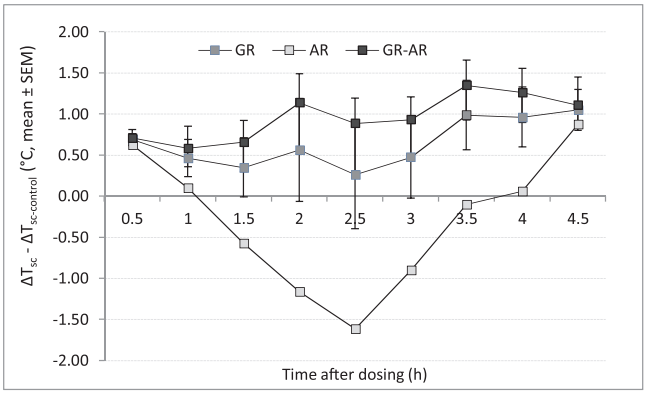


Fig. 2. Time-dose-response relationship for the quaternary mixture of PYRs.
Vehicle-control and mixture treated animals were monitored for changes in Tsc between 0.5 and 4.5 h after dosing. Three mixture doses were examined: 0.895, 4.475 and 8.950 mg total PYR/kg. A. In this panel, we show the baseline-normalized Tsc (ΔTsc) for each group. The main effects of the dose and the dose*time interaction were not significant, and there was a significant effect of time (ANOVA; $p < 0.05$). B. This panel shows how much the mean response of the highest-dose group is affected by this atypical response ("AR") of one of the animals. In panel B, we substracted the control ΔTsc from the curve of the highest-dose group shown in panel A. The data of this group are plotted with (line marked with dark gray filled squares, denoted as GR) and without (black filled squares, denoted as GR−AR) this single animal. After excluding the AR animal (including a 2.1 °C decline in Tsc from 0.5 to 2.5 h), the main effects of the dose ($p = 0.025$) and time ($p = 0.002$) were significant. Note that there was a period (i.e., between 2 and 4 h) at which the increase in Tsc over the control appears at a maximum. N = 4–6.

mixture (~8.4 mg mixture/kg, $CI_{95\%}$ = 6.1–10.7 mg/kg; unpublished data). The amount of each PYR at this intermediate mixture dose is individually subeffective to cause neurotoxicity in young adult rats (Soderlund et al., 2002; Wolansky et al., 2006; Wolansky and Harrill, 2008). In addition, the time course of effect at 8.95 mg/kg was mostly consistent with a number of neurobehavioral studies using acute oral low-dose exposure to single PYRs, a corn oil vehicle, and motor activity or core temperature monitoring devices: a mild increase in body temperature with nearly total recovery at ~4–6 h after dosing (Gordon, 2005; McDaniel and Moser, 1993; Soderlund et al., 2002; Wolansky et al., 2007a; Wolansky and Harrill, 2008).

Fig. 3 shows dose-dependent patterns of PYR distribution in tissues. Validation demonstrated that the extraction-GC-ECD analytical method was suitable for this exploratory exposure-dose-effect study. We obtained acceptable repeatability and linear correlation factors ($R^2 > 0.99$), and adequate sensitivity in the simultaneous

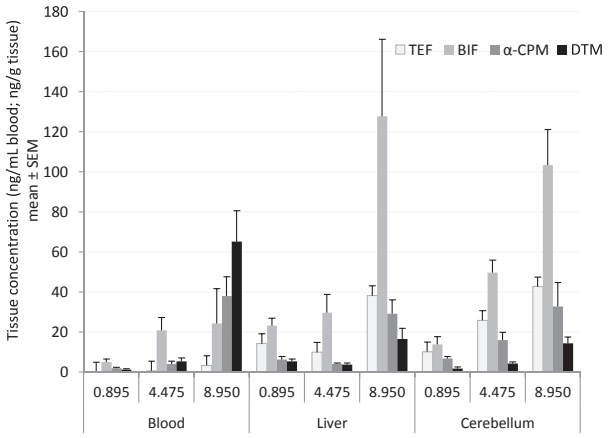


Fig. 3. Target tissue concentration.
Three PYR mixture levels were examined: a stock dosing solution (i.e., highest dose, 8.950 mg total PYR/kg) and two dilutions (i.e., 4.475 and 0.895 mg/kg total PYR/kg). PYR concentration was determined in tissues dissected out at ~6 h after oral dosing. The figure shows a greater consistency of the cerebellum compared with the other tissues examined to describe exposure-tissue-dose relationships for the PYRs coadministered. N = 4–6.

determination of all PYRs in the target tissues. The percentage recovery was within ~80–110%. The LOD was estimated between 0.3 and 1.45 ng/mL, and the LOQ ranged from 0.33 (TEF) to 1.58 (DTM) ng/mL. The CV% was the resulting computation between 1.74 (TEF) and 4.08 (BIF). PYR concentration levels were found above LOD for the majority of tissue samples extracted from the animals administered with the lowest total dose. As the dose administered increased, a trend for a dose-related accumulation of PYRs in target tissues was evidenced, predominantly in the cerebellum. In blood, liver and cerebellum, the main effects of mixture dose and PYR were statistically significant ($p < 0.0001$ in all cases). The dose*PYR interaction was also significant in all cases ($p < 0.01$); an appropriate analysis of this effect would require a more extensive schedule of the dose administered and larger sample sizes. In the cerebellum, direct relationships between the dose administered and the tissue concentration were observed for all the PYRs studied. Moreover, BIF showed trends for a greater concentration in this target tissue compared to the two cyano PYRs coadministered.

Fig. 4 shows the relationship between PYR concentration in the cerebellum and changes in baseline-normalized Tsc at 120 min, i.e., just after the onset of peak effects in the highest-dose group (see Fig. 2A–B). Although the tissue dissection time (~6 h) was a few hours after the onset of peak effects, the association between target tissue concentration and effect at 120 min was of the same strength as when using data obtained at 210 min (data not shown). In general, correlation factors were of moderate strength by performing Spearman Rank Order Correlation analyses ($p < 0.050$ in all cases, except for TEF at 210 min, $p < 0.05$). Statistically significant R factors were computed for all PYRs at 120 min. BIF and α-CYP showed the highest association factors, with $R = 0.59$ in both cases ($p = 0.008$), followed by DTM ($R = 0.52$, $p = 0.023$) and TEF ($R = 0.50$, $p = 0.031$). The similarity of results in the correlation analyses performed using effects data from 120 and 210 min is consistent with emerging evidence indicating that the burden of PYRs in the nervous system remains an adequate predictive measure of toxicity for a few hours after the period of peak effects (Scollon et al., 2011; Staar et al., 2012; Gammon et al., 2014).

Last, PYRs have similar molecular weights (i.e., ~400–500 g/mol) and lipophilicity. Likewise, the PYRs examined in this work are very similar in MW and $K_{o,w}$ (i.e., 6.0–6.6) (ATSDR, 2003). No relevant difference was found in the statistical analyses of the tissue concentration data after converting mass values in nmoles in Figs. 3, 4.

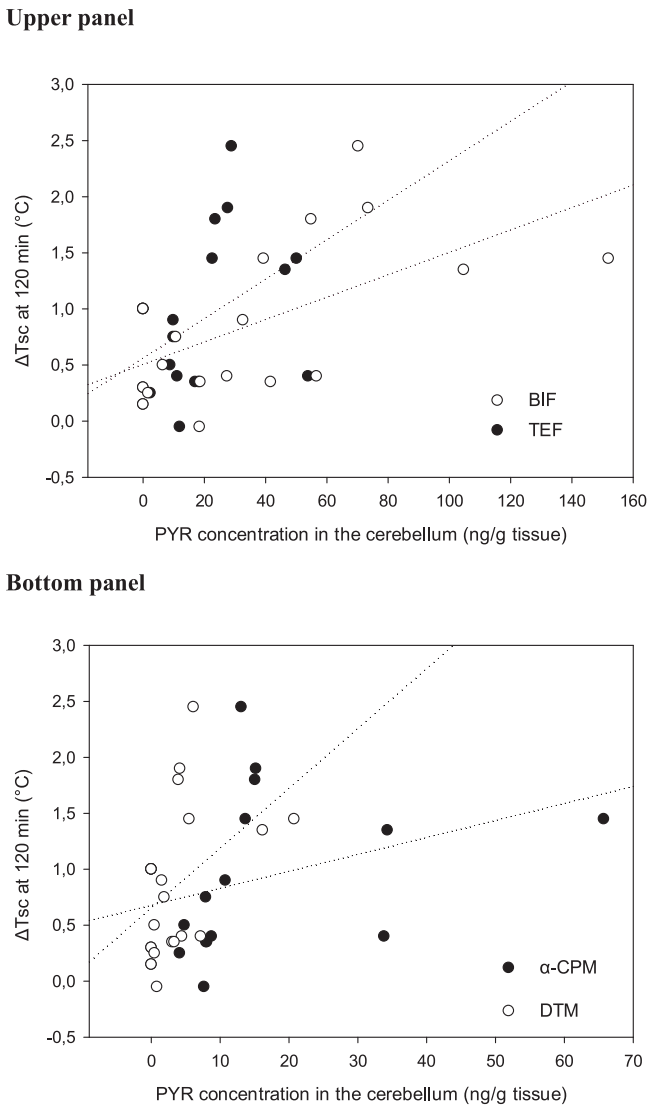


Fig. 4. Cerebellar concentration of PYR vs. changes in Tsc.
This figure shows the scatterplots for the correlation between the concentration of the noncyano PYRs TEF and BIF (upper panel) and the cyano PYRs α-CPM and DTM (bottom panel) in the cerebellum vs. baseline-normalized Tsc (ΔTsc) at 120 min. The ΔTsc means of the experimental groups administered 0.475 and 0.895 mg/kg were nearly identical at this time, suggesting that it was a toxicologically relevant time point for maximal effect (Fig. 2). Correlations at t = 120 min were significant in all cases ($p < 0.05$).

4. Discussion

This research studied the relationship between the dose administered, the internal dose, the target tissue concentration and the effects using an index measure of body temperature after oral low-dose exposure to a quaternary mixture of PYRs. The route of exposure, the vehicle, age and body weight of the experimental animals were similar to those used in previous single compound and mixture studies (Scollon et al., 2011; Starr et al., 2014, 2012, Wolansky et al., 2009, 2007b, 2006). We tested a mixture with two qualitative differences compared to the two PYR mixtures previously examined (Starr et al., 2012; Hughes et al., 2016a; Wolansky et al., 2009; Wolansky et al., in preparation). First, all mixed chemicals were a set of the most potent PYR preparations, mostly consisting of *cis* isomers (Wolansky and Harrill, 2008). Thus, *cis*-isomer specific pharmacokinetics may have driven most of the detoxifying biotransformation of PYRs over time; a combination of isomer-rich and racemic compounds was used in previous studies. Second, we used a body temperature endpoint responding differentially according to the PYR type; all previous single compound and mixture studies reported a dose-dependent decline in motor activity independently of the chemical structure of the PYRs examined (Hughes et al., 2016a; Starr et al., 2014, 2012; Wolansky et al., 2009).

The available evidence on PYR effects in rats reveals to what extent the single compound time-dose-effect patterns of acute neurotoxicity contributed to the mixture effects observed in our work. BIF is reported to cause dose-dependent increases in rectal temperature (Wolansky et al., 2007b). In the pilot work, TEF doses of 0.1–6 mg/kg were ineffective in causing an evident increase in Tsc, but we were unable to use TEF doses ≥ 9 mg/kg in formal dose-response assays because a more robust alteration in Tsc would have been soon followed by a lethal syndrome. CPM and DTM may cause primarily hypothermia at high-effective doses, although a mild increase in the core temperature occurs during the initial ~30–120 min after administering low-to-middle effective doses of these cyano PYRs (McDaniel and Moser, 1993; Wolansky et al., 2007a). The time of peak effect for individual PYRs is 1.5–4 h using a corn oil vehicle, 1 ml/kg dose volume and motor activity as an endpoint (Soderlund et al., 2002; Wolansky et al., 2007b, 2006; Wolansky et al., in preparation). The mixture tested in our work caused a clear increase in normalized Tsc over the control beginning at 30–60 min, an alteration still noticeable at 4.5 h post-dosing (Fig. 2A–B). This time-related trend appears to be consistent with the monotonic increase in body temperature reported in studies of various noncyano PYRs (Soderlund et al., 2002; Wolansky and Harrill, 2008) and the biphasic response observed after exposure to various cyano PYRs such as DTM and CPM (McDaniel and Moser, 1993; Wolansky et al., 2007a). The time interval where Tsc increase remained at a maximum was also consistent with the time-response relationship observed in the mixture study of Staar et al. (2012) using motor activity as an endpoint. Moreover, the extended Tsc increase over control may be considered consistent with the prolonged tremorigenic activity reported after exposure to BIF (Holton et al., 1997; Scollon et al., 2011; Soderlund et al., 2002; Wolansky et al., 2007b, 2006). However, in our work the stock mixture solution consisted of very low concentrations of the single PYRs (Wolansky and Harrill, 2008). More surprisingly, the intense hypothermic action observed in one animal of the highest dose group (i.e., reducing 2.1 °C its body temperature along a 2-h period; Fig. 2B) seems to suggest that the susceptibility among individuals may differ in the strength as well as in the quality of the clinical response after acute exposure to PYR mixtures.

Absorption of PYRs mostly by simple diffusion after single oral exposure in rats occurs relatively fast. Up to 60–70% of an ingested dose may enter the body through the gastrointestinal epithelium in mammals; a ~10–20% fraction of the dose administered may remain intact in feces (Tornero-Velez et al., 2012a, 2010). In general, PYRs dissolved in corn oil reach concentration peaks in circulating blood at 2–4 h after dosing. Moreover, the oral bioavailability of PYRs may be very variable, i.e. ~14–60%. It has been suggested that efflux transporters along the intestinal tract limit the absorption of these chemicals (USEPA, 2007). In addition, once an internal dose of PYRs is distributed into the tissues, elimination from the brain is slower than from blood and liver. The $t_{1/2}$ of PYRs in the brain after single acute oral dosing is generally a few hours regardless of the isomer composition of the compound examined. Noteworthy, tissue residues of the *cis* isomers may remain there for a longer time than the *trans* isomers. In blood and liver, a low dose of PYR is eliminated much faster ($t_{1/2} < 1$ h). After acute exposure to a low dose of α-CPM it remains longer in blood and is eliminated slower by urine than CPM in experimental animals and humans, though only a minor portion of the body burden is found in the brain (CDPR, 2016; Tornero-Velez et al., 2012a). Most relevant to our work, preparations with the most potent *cis* isomers may potentially reach larger peak concentrations in target tissues at ~4–6 h after dosing than equivalent exposure levels of the *trans* isomers or the racemic sample (Tornero-Velez et al., 2012a). In our study, most of the test mixture consisted of

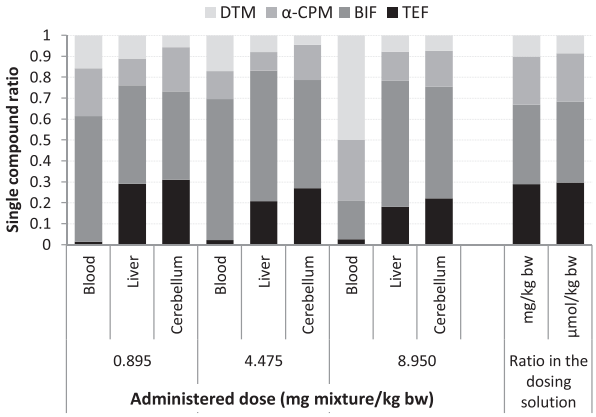


Fig. 5. Concordance between mixing ratios in the dosing solution and target tissues.

Mass/mass relative contribution of each individual compound to the global PYR burden in blood, liver and cerebellum tissues at the three mixture doses examined. The last bar on the right illustrates the mixing ratios of PYRs in the solution administered. Note that the cerebellum and, to some extent, the liver show the best matches for single PYR proportions compared to the test mixture composition. Single compound proportions in the dosing solution (extreme right) are shown as mass/mass and µmol/µmol ratios for an illustration of the absence of relevant molecular weight dependence at any comparison of the mixing ratios in the dosing solution and compound proportions in target tissues.

cis isomers, so the concentration in the cerebellum of the PYRs examined at the time we dissected out tissues may have been nearly or slightly below peak concentrations (see also Gammon et al., 2014).

In our work, the similarity of MW and $K_{o,w}$ between the PYRs examined strongly suggests that their accumulation in the nervous system would be hardly influenced by these features. Accordingly, it may be postulated that the nearly equitoxic doses of the PYRs combined in a mixture would be globally "seen" by the brain as if these were roughly the same. If this interpretation were correct, the total brain burden of PYRs (in nmol) would be expected to have a strong correlation with the mixture dose administered. Figs. 5 and 6 support this interpretation. Fig. 5 explores the equivalence between the mass/mass mixing rates in the dosing solution and the relative concentration of the PYRs in the target tissues. This figure shows that the best match for the PYR mixing

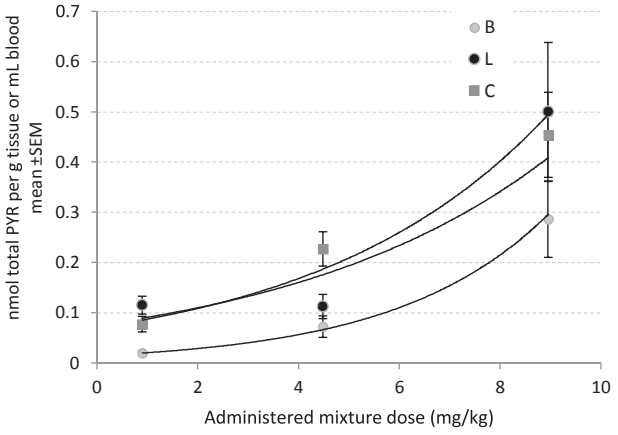


Fig. 6. Correlation between the mixture doses administered and total PYR in the cerebellum.

The concentrations of all the test compounds in nmol/g tissue (cerebellum, C; liver, L) or nmol/mL (blood, B) were summed to evaluate the association between the global PYR burden in the target tissue and the oral mixture dose administered (i.e., 8.950, 4.475 and 0.895 mg total PYR/kg). Note that the lipid-rich tissues liver (L) and cerebellum (C) show greater concentrations of PYRs than blood (B). All correlation tests were significant ($p < 0.0002$), and the association was most robust for cerebellum ($p < 0.00001$).

Table 2

Comparison between the findings in the BIF study of Scollon et al. (2011) and BIF disposition in our work. Both studies used similar test material, dosing conditions and dissection time, making possible a comparison of BIF distribution in target tissues to explore the influence of coadministered PYRs in BIF disposition.

Study	Scollon et al. (2011)	This work
Dosing conditions	Corn oil vehicle, 1 mL/kg dosing rate, high purity BIF sample, similar animal age and bw range	
Rat strain	Long Evans	Hsd:WI Wistar
Tissue dissection time after dosing (h)	~5.5[a]	~5.5–6.5[a]
Dose of BIF (mg/kg)		
Single compound study	2–4	N/A
Quaternary mixture study	N/A	3.4 (see Table 1)
Target tissue	Whole brain	Cerebellum
Blood concentration (ng BIF/mL)	~20–120[b]	24.3 ± 17.3, mean ± SEM[c]
Target tissue concentration (ng BIF/g)	~40–240[b]	103.5 ± 17.6, mean ± SEM[c]
Brain/blood ratio	~2[b]	~4, mean ± SEM[c]

[a] The euthanasia, followed by tissue dissection, was conducted soon after finishing the MA (Scollon et al., 2011) or Tsc (this work) monitoring assays, taking ~1–1.5 h per experiment. So, the exact times between dosing and sacrifice may differ up to a maximum of ~30–45 min across experimental animals.

[b] Value estimates based on a visual analysis of results in Scollon et al. (2011).

[c] Values based on actual data from experimental animals.

rates in the test mixture was that found in the cerebellum regardless of the dose administered. Moreover, the relative concentrations of all PYRs in the liver were fairly similar to the composition of the mixture dosing solution. These comparable trends may have been due to the highly lipidic nature of the brain and liver tissues. In addition, Fig. 6 shows that the total nmolar concentration of PYRs in the target tissues was highly correlated to the mixture dose administered, especially in the cerebellum (Pearson's Correlation $r = 0.89$, $p < 0.000001$).

We were also interested in exploring whether the disposition of BIF in a single-compound study was consistent with the blood absorption and brain concentration of the same PYR when coadministered with other PYRs, using similar test materials, dosing conditions and tissue dissection times. Table 2 compares the experimental conditions and disposition results in the study of BIF carried out by Scollon et al. (2011) and those of our work. It should be kept in mind that two different rat strains and animal colonies were used in these studies. We do not know to what extent the strain-related physiological and pharmacokinetic diversity reported for some chemicals (Oltra-Noguera et al., 2015) may influence the variability within and between treatment groups in exposure-dose-effect assays for PYRs (see also Wolansky and Tornero-Velez, 2013). In our research, we determined PYR concentrations in target tissues at ~6 ± 0.5 h after dosing, i.e., ~1 h later than one of the dissection times in the study by Scollon et al., and BIF was the PYR with the highest concentration in the cerebellum (Fig. 3). In the BIF studies of Scollon et al. (2011) and Gammon et al. (2014), the decline in the slope of the relationship between the dose administered and the blood concentration occurred at longer times, but the blood level decreased slightly and the brain concentration showed no evident change within the 4–8 h interval. Consequently, brain/blood ratios steadily increased from 4 h on after dosing of BIF. More recently, Hughes et al. (2016b) conducted a TK study of 0.3–3.0 mg BIF/kg. The 3-mg/kg dose and the 6-h testing time were similar to the BIF dose and dissection time used in our work, facilitating a direct comparison of the target tissue concentrations. In rats, the oxidative pathway is much more active than the ester hydrolysis biotransformation of BIF (Gammon et al., 2012). In turn, these toxicokinetic properties may result in a rapid action of metabolism and distribution, which greatly

facilitates the clearance from blood faster than the removal of a BIF burden accumulated in lipid-rich target tissues. Even a little difference in dissection times (i.e., ~30–60 min longer time for sacrifice in our work than in the study of Scollon et al., 2011) would provide an extended time for BIF clearance from blood, although the concentration in the brain would be barely affected (Hughes et al., 2016b; Gammon et al., 2014; Scollon et al., 2011). Thus, the relationships between the dose administered, blood dose and nervous system dose observed in our study (Figs. 3, 4) are consistent with the results of the above-mentioned TK studies, globally suggesting a similar disposition of BIF in single-compound and mixture studies using young adult rats and low-dose acute oral exposure schemes.

Another difference between our work and previous single compound and mixture studies of PYRs using comparable dosing conditions (Scollon et al., 2011; Staar et al., 2012, 2014; Gammon et al., 2014; Hughes et al., 2016a,b) is that the nervous system region selected for PYR determination was different (cerebellum vs. whole brain). The cerebellum featured as a reliable target tissue to characterize exposure-dose-effect relationships for PYRs. We estimated high correlation factors, suggesting a strong association between the concentration of each PYR in the cerebellum and the mixture dose administered ($R^2 \geq 0.95$, data not shown; Fig. 6). Thus, our results and previous studies strongly suggest that BIF uptake into the brain is not greatly influenced by concurring exposure to other PYRs, provided that the doses of the single compounds administered and the total mixture dose are near-threshold for acute oral neurotoxicity.

The extrapolation of our findings to humans is limited by various sources of uncertainty. First, although the test mixture structuring included environmental data (Tulve et al., 2006, 2011; Jardim and Caldas, 2012; Morgan, 2012), one co-occurrence analysis (Tornero-Velez et al., 2012b), and was aimed to simulate a combination of near-effective doses of the most toxic PYR isomers, it did not actually reflect a realistic worst-case scenario of exposure to PYRs. As a point of reference, it is estimated that the daily exposure to permethrin is a few μg/day in humans (i.e., a $\sim 10^1$–10^2 ng/kg dose for a 60-kg adult; ATSDR, 2003), but we determined a total PYR burden of ~120–290 ng/g cerebellum at the ~9 mg/kg dose (Fig. 6). Second, the oral (i.e., bolus) procedure used in our study is not directly comparable to an oral diet, dermal or hand-to-mouth patterns of human exposure to pesticide residues (Zartarian et al., 2012; Saillenfait et al., 2015). Third, the time-of-peak-effects of the PYRs examined were not exactly the same. Previous work using motor activity or intraperitoneal body temperature as an endpoint (Wolansky et al., 2007a,b; 2009; Wolansky and Harrill, 2008) and our single-compound pilot assays using the subcutaneous temperature showed up to 2-h difference in the peak-effect time interval. We determined target tissue concentrations of all PYRs at only one dissection time (~6 h after dosing), i.e., soon after animal Tsc started to return to the vehicle-control response (Fig. 2A–B). Previous TK studies allow assuming a lower brain/blood ratio for tissue concentrations at dissection times earlier than the one used here. Moreover, the time for peak concentration (T_{max}) in the brain after acute oral dosing may be dependent on the PYR (Kim et al., 2007; Scollon et al., 2011; Staar et al., 2012; Hughes et al., 2016b); and for a same PYR, different cerebral regions may differ up to 3 times in T_{max} (Anadón et al., 1991). Hence, we do not know how dependent the exposure-dose-effect relationships of the individual PYRs in the mixture studies are on the diversity of peak-effect times and target tissue T_{max} that may be observed for PYRs in single compound studies. Comprehensive pharmacokinetics-effect models will thus be required to test how blood dose, disposition into target tissues and joint actions after low-dose exposure to individual or combined PYRs in rats compare to health risks of toxicity posed to humans after aggregate exposure to environmentally relevant combinations of PYRs.

4.1. Conclusion

In conclusion, our results are consistent with previous data (Hughes et al., 2016a; Starr et al., 2014, 2012), remarking the toxicological relevance of near-threshold doses of PYRs under cumulative exposure conditions. This and previous studies in rats strongly suggest that occasional, intermittent or daily combined exposure to pesticide products containing isomer-rich or racemic PYRs resulting in single PYR levels of $\geq 10^{-1}$ nmol/g brain may cause alterations in vital mechanisms such as the control of the body temperature.

5. Disclaimer

Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

6. Competing interest declaration

The authors have no competing interests.

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