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By Indri Santiasih

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The Metabolites Resulting from Particulate Matter Exposure of Prallethrin and *d*-Phenothrin Mixture in The Inhalation Exposure Chamber

Indri Santiasih[1*], Harmin Sulistiyaning Titah[2], Joni Hermana[2]

[1]Study Program of Risk and Safety Engineering, Department of Marine Enginering, Politeknik Perkapalan Negeri Surabaya, Keputih, Sukolilo, 60111, Surabaya, East –Java, Indonesia

[2]Department of Environmental Engineering, Faculty of Civil, Planning and Geo Engineering, Institut Teknologi Sepuluh Nopember, Keputih, Sukolilo, 60111 Surabaya, Indonesia

Email: indri.santiasih@ppns.ac.id

Abstract. Prallethrin and *d*-phenothrin mixture were active ingredients in mosquito repellents (MR) and were neurotoxic to mammals. This study examined the role of prallethrin and *d*-phenothrin mixture as particulate matter generating toxic effects in an inhalation exposure of mice that produced the metabolites. The inhalation exposures were conducted by blowing the solution with an airflow rate of 4 L/min through a diffuser to generate PMs. The dose 1 was a mixture of prallethrin 0,001 mg/L dan *d*-phenothrin 0,00125 mg/L, while dose 2 was a mixture of 0,000624 mg/L dan *d*-phenothrin 0,005063 mg/L. The parameters including body weight, PM, and carboxylesterase (CE) concentration were monitored every 20 days during 60 days of observation. The simultaneous interaction between doses and temperature variation generated important effects on CE concentration. They also provided a significant effect on the *d*-phenothrin residue, however, they had no consequence on the prallethrin residue. Benzoic acid, 2-benzoyl-, methyl ester was believed as the metabolites on inhalation exposure of prallethrin and *d*-phenothrin mixture in the mouse liver. This study suggests that PMs generated were dominated by ultrafine particles. It also proved that PM results were hazardous in terms of inhalation exposure according to CE concentration, and the accumulation of residue and metabolite in the mouse liver.

Keywords: particulate matter; prallethrin; *d*-phenothrin; pyrethroid; metabolite; inhalation exposure.

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Introduction

Prallethrin and *d*-phenothrin are pyrethroid family and common ingredients of mosquito repellents (MRs). The previous study found that they produce neurotoxic with sodium channels being their well-established target site in mammals [1]. Recently, MR containing two, three, or even four pyrethroid compounds have been preferred to obtain adverse effects as an insecticide in the market. MRs either in the form of mats, sprays, or coils significantly generate particulate matters (PMs) [2,3] and there is evidence to suggest that indoor PM exposure produces health disorders [4,5]. It is essential to consider seriously since people spends most of their time (80%) indoors [6].

PMs are a heterogeneous complex mixture between solid and/or liquid [7] which changes in time and space. The size and chemical composition of PMs are important parameters in several processes occurring in the atmosphere [8]; for instance, particle growth and gas-particle interactions [9]. Kulkarni *et al.* [7] investigated particle size, which has an important role in determining particle behavior. Particle properties that improve adverse health effects depend on their size, specific surface area, number, and chemical composition that determine the toxicity of any specific element. Owen *et al.* [10] investigated the health effects due to indoor PMs related to the diameter and mass of particles. When the particle size is reduced from micrometer to nanometer range, increasing toxicity appears due to the increase in particle surface area [11].

Carboxylesterase (CE) was α/β fold hydrolases that hydrolize a catalytic series of serine, histidine and aspartate acid or glutamic acid residue [12]. CEs can be found in mammals organs/tissues and the highest activity is in the liver [13]. CEs contribute to hydrolyze ester, thioester, amide which were contained in xenobiotic and endogenous substances [14], even though when multiple isomers are mixed into a compound, they illustrate stereo -/enantioselective hydrolysis to certain isomeric. This enzyme plays an important role in pyrethroid biodegradation, thus, pyrethroid comprises low to moderate toxicity and is generally secured in oral exposure to mammals [15]. Stok *et al.* [16] examined stereoisomers which are found in almost all types of pyrethroid insecticide. The isomer differences of the same pyrethroid show crucial differences in hydrolysis resistance in the same CE isozyme and hydrolytic toxic stability contributes to toxicity differences. The hydrolysis process produces parent chemical residue and intermediate compounds (metabolite) resulting from breaking the covalent bond. The covalent bond dissection is conducted to obtain a smaller molecular weight of the compound, therefore, it can be excreted easily through urine. In the continuous exposure system, the xenobiotic exposure is provided continuously exceeds CE capacity for hydrolysis. Consequently, there is a number of residues and/or metabolites that accumulate in the organs/tissues and are suspected of generating health disorders [17]. Hughes *et al.* [18] examine the disposition of residue from a mixture of b-cyfluthrin, cypermethrin, deltamethrin, esfenvalerate and cis- and trans-permethrin in rat. They are orally administered in corn oil at seven dose levels and found the lowest concentration in the blood are trans-permethrin and b-cyfluthrin and in the brain are deltamethrin and esfenvalerate.

However, a study examining the MR compounds mixtures as a particulate matter exposure on the mouse and the toxic effects generated have not been studied, even though MR as a single compound has been investigated by researchers. These compound mixtures are predicted to produce different metabolites with those previously examined. Hence, it is very important to investigate the toxic effects of prallethrin and *d*-phenothrin mixture through inhalation exposure. This study is aimed to examine the toxic effect of prallethrin and *d*-phenothrin mixture through inhalation exposure as particulate matters that predicted generating the new metabolite in the mouse liver.

Materials and Method

Animal husbandry and Treatment

Six-week-old male BALB/c mice were obtained from the Pharmacology Laboratory of Airlangga University and acclimatized for 14 days. The room was maintained at 30.3°C ± 1.4°C with relative humidity (RH) of 63.08% ± 2. 87% and light: dark cycles of 12:12 hours. The 12 mice were housed together in stainless steel wire mesh cages (W 350 mm × L 400 mm × H 180 mm), *ad libitum* provided for tap water and a commercial diet from PT. Charoen Pokphand Indonesia. After acclimatization, three mice were placed into a chamber for exposure to a prallethrin and *d*-phenothrin mixture exposure for four hours a day during 60 days of

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observation. The mice were euthanized every 20 days by giving an injection of ketamine 100 mg/kg body weight and xyla 5 mg/kg body weight. After sacrificing, the liver was collected, added liquid nitrogen, and stored immediately at -80°C to analyze carboxylesterase and pyrethroid residue. The study was approved by the Animal Care and Use Committee (ACUC) of the Veterinary Faculty of Airlangga University with certificate number 716-KE.

Chemicals and Reagents

Prallethrin and *d*-phenothrin were obtained from Sigma Aldrich with a catalog number of prallethrin 32917 and *d*-phenothrin 36193. Two doses were varied namely dose 1 and dose 2 according to NOAEL value of each compound of 28 days' exposures by WHO [19]. Dose 1 was a mixture of prallethrin 0,001 mg/L and *d*-phenothrin 0,00125 mg/L, whereas dose 2 was a mixture of prallethrin 0,000624 mg/L and *d*-phenothrin 0,005063 mg/L). These active ingredients were diluted several times to obtain the preferred concentration. Doses were provided for each mouse.

Experimental Groups of Inhalation Exposure

The experimental groups were divided into four groups, namely groups A, B, C, and D according to dose and temperature variations. The temperature was varied at 25°C (temperature 1) and 31°C (temperature 2). Group A was the experiment with exposure of dose 1 and temperature 1, accordingly group B was with the exposure of dose 2 and temperature 1, group C was with the exposure of dose 1 and temperature 2, and group D was with the exposure of dose 2 and temperature 2. The control groups were divided into two groups, namely negative control (NC) and positive control (PC). NC was a group without any treatment at all and PC was the experiment with exposure to solvent only. There were three chambers for replication for each group, and each replicate contained three mice as illustrated in Figure 1.

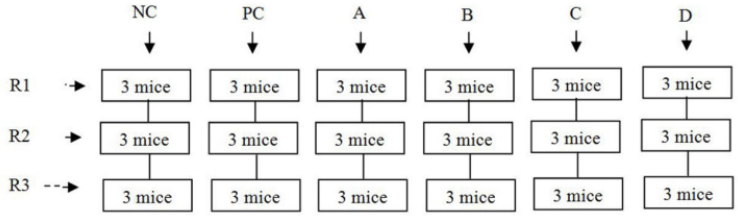

Figure 1. Experimental Groups of Inhalation Exposure
NC: negative control group; PC: positive control group; R: replication.

Purification of Carboxylesterase, The Metabolite, and Residue of Pyrethroid

Carboxylesterase was analyzed using an enzyme-linked immunosorbent assay (ELISA) of Mouse Carboxylesterase 1 (Ces1). After harvesting, the mouse liver was washed with phosphate buffer saline pH 7.2 containing 137 mM NaCl; 2.7 mM KCl; 10mM Na_2HPO_4; 2 mM KH_2PO_4 dan Tris Base-HCl, then succeeded frozen immediately by adding liquid nitrogen and maintained at -80°C. Protein extraction was referred to Borovikova [20] and executed by adding 2.5 mL extraction buffer containing 1% Triton X-100 (Sigma Aldrich) and protease inhibitor cocktail (Sigma Aldrich PB340) into 0.1 g mouse liver, followed by sonicated for three minutes, took a break for a minute and then sonicated for a second time for three minutes. The homogenate was centrifuged for ten minutes at 12000 rpm, afterward, the supernatant was obtained to analyze using ELISA kit Mouse Carboxylesterase 1 (Ces1) ELISA Kit from Bioassay Technology Laboratory with a catalog number of E2395Mo. CE readings were conducted using ELISA reader GloMax Explorer from Promega Corporation (USA) in Tropical Disease Center of Airlangga University. All equipment and apparatus used were already calibrated.

Pyrethroid metabolite and residues were determined using gas chromatography-mass spectrum (GC-MS), and the sample purification referred to Barbini *et al.* [21]. The sample purification was carried out using three types of reagent, namely reagent 1, reagent 2, and reagent 3. Reagent 1 contained acetone – petroleum ether in a ratio of 1:1 (v/v), reagent 2 was acetonitrile-dichloromethane in a ratio of 3:1 (v/v) and reagent 3 included n-hexane –diethyl ether in a ratio of 3:2 (v/v). The homogenization was conducted by adding EDTA 2 mM and 5 mL reagent 1, centrifuged 3000 rpm at 2°C for 15 minutes. After that, the homogenate was transferred into another tube through an anhydrous sodium sulfate layer (Na_2SO_4) to remove water and organic contents. Then, the homogenate overflowed into another matrix containing 2 mM and 5 mL reagent 1, centrifuged 3000 rpm at 2°C for 15 minutes. Clean-up was carried out by

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dissolving homogenate into two times of 2 mL reagent 2. They were mixed for 15 minutes at 12°C. The supernatant was spilled out into a new tube and added two times of 2 mL reagent 2, then mixed for ten seconds. Extract combination centrifuged 3000 rpm for 15 minutes at 12°C. The final supernatant was dissolved into two times of 2 mL n-hexane and ready to be injected into GC-MS.

Measurement of Carboxylesterase (CE) Concentration in Mouse Liver

CE concentration was verified using Mouse Carboxylesterase ELISA kit from Bioassay Laboratory manufacturing, Shanghai-China. The standard curve was depicted using Curve-Expert Software with the five gradient concentration variation of 48 ng/mL, 24 ng/mL, 12 ng/mL, 6 ng/mL, 3 ng/mL, 1.5 ng/mL, and blank, to obtain the standard equation. The standard equation was occupied to determine CE concentration [22].

The validation method of CE concentration was carried out by using the validation parameter of robustness, precision, and trueness [23]. The parameter of robustness was conducted to certify that the measured concentrations do not depend on the changes, It was adjusted by adding temperature intervals of 25°C ± 0.3°C; 25°C ± 0.6°C; 25°C ± 0.9°C (temperature 1), and the temperature 2 of 31°C ± 0.3°C; 31°C ± 0.6°C; 31°C ± 0.9°C into the protocol. The parameter of precision confirmed the closeness of agreement between independent test results obtained under stipulated conditions. It was executed by comparing the test result with the standard curve. The parameter of trueness certified that the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The reference value was directly derived from certified reference material (CRM) [23].

Measurement of Mouse Body Weight

The mouse body weight was monitored every day using a digital counting scale device of Sartorius ENTRIS 4202-1S from Sartorius manufacturing to observe the health condition of mice prior to the treatment and to observe the treatment effect on mice.

Measurement of Temperature and Relative Humidity (RH)

Temperature and RH were evaluated using a Son off TH Sensor AM2301 from ITEAD (China), in every five minutes for four-hour exposures during 60 days of observation and monitored using an android system and automatically reserved in the data logger.

Measurement of Particulate Matter (PM)

PMs were measured at the third hour of exposure in the breathing zone of mice using an Aerocet 531S Particle Mass Profiler and Counter from Met One Instrument Inc (USA). Readings were taken at 7 cm in height from the bottom of the chamber to represent inhaled PMs.

The analytical validation method of PM measurements was conducted using standard solutions in three doses (A, B, and C). The concentration ratio of prallethrin and *d*-phenothrin mixture was 1:1. Dose B was a mixture of prallethrin and *d*-phenothrin based on NOAEL value, dose A contained 10 % of dose B, whereas dose C was ten times larger of dose B. The analytical validation data of PM concentration was carried out by evaluating the PM concentration of two measurement devices, a High Volume Air Sampler from Sole Agent HI-Q Environmental Products Company manufacturing and an Aerocet 531S Particle Mass Profiler and Counter from Met One Instruments Inc. manufacturing [24]. The PM in size of 2.5 µm was selected in the present study to investigate the performance of the methodology adopted [25], regarding the criteria of linearity, precision, and accuracy. The results of each measurement were compared.

Experimental Set-Up

Prallethrin and *d*-phenothrin were dissolved in acetonitrile and then diluted several times in distilled water [26], then employed for inhalation exposure to tested mice. The solution was volatilized using a diffuser, in which the air supply was derived from an air pump (RC-Q6) obtained from Adam Aquarium manufacturing, and discharged at 4 L per minute into the solution [27] to produce aerosols. The aerosols were inserted into the whole-body exposure chamber which contained three mice. The chamber condition was not well mixed in order to represent the real conditions of poor indoor air circulation in a modern room. Duration exposures were four hours a day [19] during 60 days of observation of prallethrin and *d*-phenothrin mixture. The experiment was executed at temperatures and dose variation in a continuous system chamber. The exposure technique described was already calibrated.

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Identification of The Prallethrin and *d*-Phenothrin Mixture Metabolite using Gas Chromatography-Mass Spectrometry

The analytical conditions were as follows: capillary column Agilent 19091S-433: 93.92873 HP-5MS 5% Phenyl Methyl Silox; temperature range 0 °C—32[illegible]C (325 °C); dimensions 30 m x 250 µm x 0.25 µm; temperature set-up: initial 80°C; pressure 11[illegible]1 psi; flow 1.2 mL/min; average velocity 40.494 cm/sec; run time [illegible] min; post-run time 0 min; oven: equilibration time 0.25 min, maximum temperature 325°C; initial 80°C; hold time 1 min; post-run 70°C; program: rate 10°C/min; value300°C; hold time 10 min; front injector: syringe size 10 µL; injection volume 1 µL; analytical mode, selected ion monitoring (SIM), library: NIST 17.0.

The data validation was conducted by analyzing using standard solutions in three doses (A, B, and C). The concentration ratio of prallethrin and *d*-phenothrin mixture was 1:1. Dose B was a mixture of prallethrin and *d*-phenothrin based on NOAEL value, dose A contained 10 % of dose B, whereas dose C was ten times larger of dose B. Accuracy (bias) and precision were estimated from the analysis of quality control (QC) samples at low nearly the lower limit of quantification (LLOQ)) and high concentration, in three replicates for each concentration refer to Mannocchi *et al.* [28]. The acceptance criterion for bias was within ±15% of nominal value (±20% close to LLOQ), for precision was within ±15% relative standard deviation (RSD) (20% close to LLOQ). The LOD (Limit of Detection) was determined by the analysis of spiked samples with a declining level of concentration of the analyte. The LOD value of signal-to-noise ratio equal to or greater than three was selected. The LLOQ was determined by the analysis of fortified samples with a decreasing level of concentration of the analyte. For LLOQ a value of signal-to-noise ratio equal to or greater than ten was preferred. Recovery was calculated by analyzing extracted spiked samples at high and low concentration in relationship with the curve calibration, compared with the control samples [28].

Statistical Analysis of Particulate Matter Concentrations

All data series were analyzed using SPSS 21.0. The normality was determined with a Kolmogorov-Smirnov test. One-way ANOVAs were conducted to determine differences between groups, whereas independent t-tests were used to analyze differences between the two groups.

Results

Animal Body Weight

Bodyweight (BW) was measured to observe the exposure effects of the MR active substance to tested animals and also illustrates the clinical indicator of animal health. BW of NC group indicates a greater increase in weight as compared with other groups, *i.e* the average increased of 44.5% at day 20 to day 40, subsequently declined of 18.2% at day 60 that contrast with day 40.

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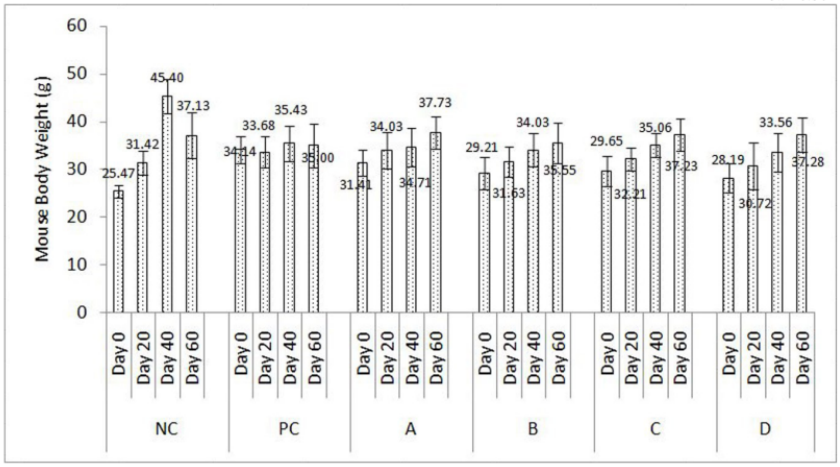

Figure 2. Animal Body Weight

The PC group encompassed a static development of BW and relatively contained a slight increase and decrease as well. The treatment groups of A, B, C, and D illustrated a similar pattern of BW on all days of measurements (Figure 2), with the results of statistical analysis indicating no significant differences of BW between groups (p-value = 0.919).

Temperature and Relative Humidity (RH)

Temperature and relative humidity (RH) played an important role in determining particle behavior. In the treatment group, the temperature was varied into 25°C dan 31°C in the treatment group of A, B, C, and D, and successfully conditioned in accordance with research variables (Figure 3). The temperature in the NC and PC group were affected by outdoor temperature with the lowest of 31.5 °C ± 0.4 °C (NC group) and the highest of 32.8 °C ± 0.6 °C (PC group). Likewise, the RH was heavily influenced by the bubble-making process to provide a saturated point.

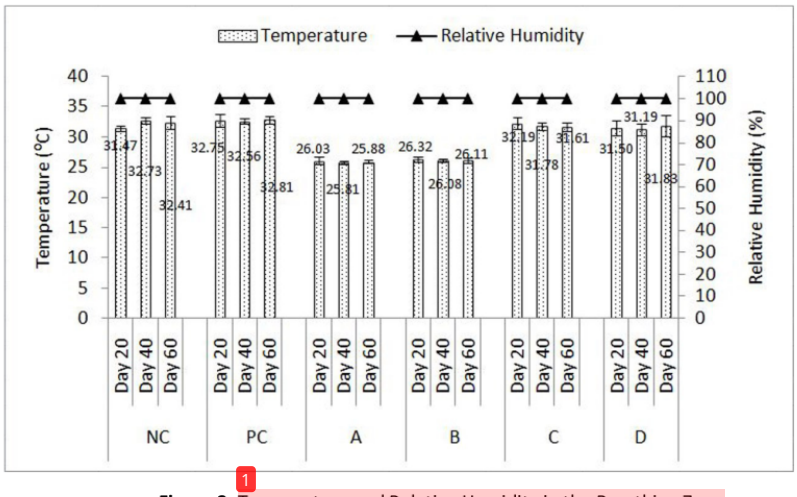

Figure 3. Temperature and Relative Humidity in the Breathing Zone

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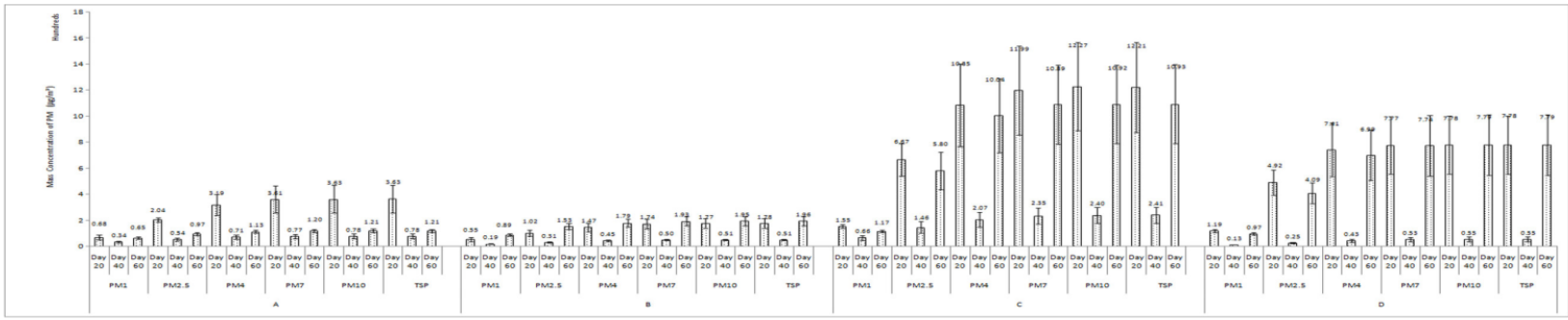
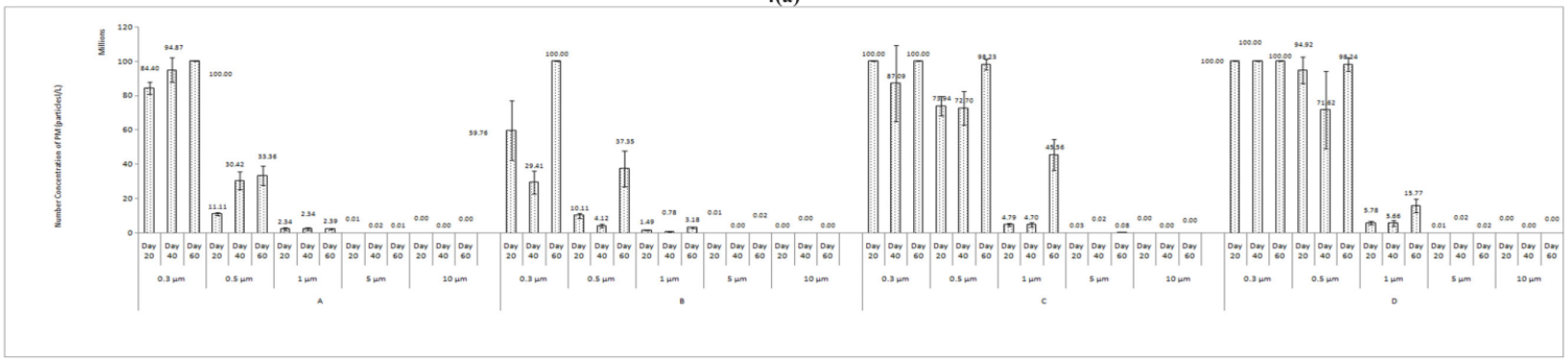


4(a)



4(b)

Figure 4 Mass Concentration (a) and Number concentration (b) of PMs at different days and groups in the breathing zone. Data are presented as means ± standard deviation (SD).

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Mass Concentrations of Particulate Matters

Figure 4 illustrates that the group of C and D (groups with a temperature variation of 31°C) relatively contained greater mass concentration compared with A and B groups (groups with a temperature variation of 25°C) within the same dose. The greatest increase was the mass concentration of group C. In the lower temperature (25°C), the group that showed the lowest mass concentration was group B.

Number Concentrations of Particulate Matters

PM number concentration in the breathing zone (Figure 4b) shows a polydispersed distribution with the major contribution being ultrafine PMs (diameter of ≤ 1 µm). They contained the largest concentration in a diameter of 0.3 µm, followed by 0.5 µm, 1 µm, and 5 µm. The same patterns occurred throughout all daily measurements for the 20th, 40th, and 60th days. Particles of 5 µm in size approached zero, and even particles 10 µm were not measurable.

Carboxylesterase (CE) Concentration

CE concentration of negative control developed according to age maturity, while CE in positive control was relatively constant growth at day 20th, 40th and 60th (Figure 5). Groups with dose 1 variation (A and C) indicated lower CE concentration compared to groups with dose 2 variation (B and D). CE concentration was an enzyme response that directly proportional to the increase or decrease of the substrate (prallethrin and *d*-phenothrin).

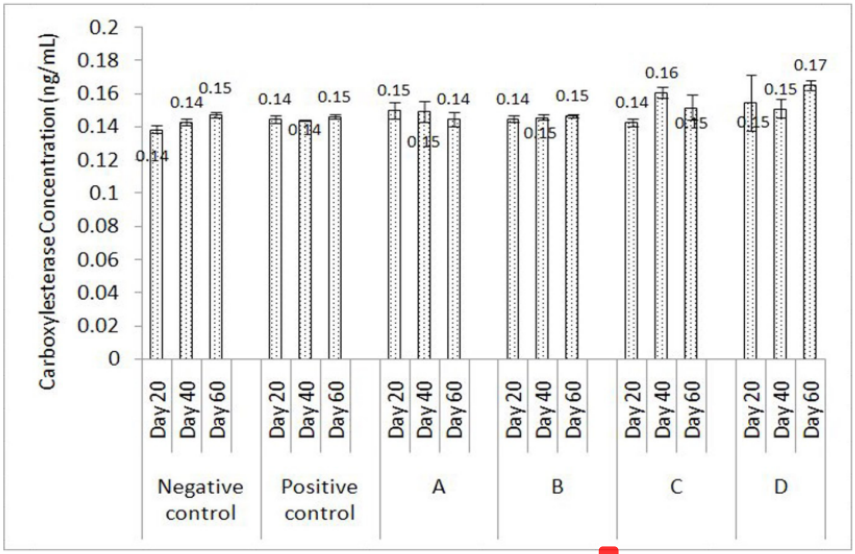

Figure 5. Carboxylesterase Concentration in The Experimental Group. Data were presented as means ± standard deviation (SD).

The Metabolite and Residue of Prallethrin and *d*-Phenothrin Mixture

The metabolites of prallethrin and *d*-phenothrin mixture that were found as well in this research besides the residue of parent chemicals were presented in Table 1.

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Table 1.The Metabolites in The Mouse Liver

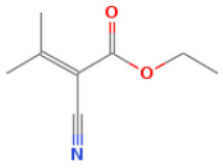
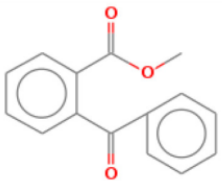
Names of Compounds	Molecular Weight (g/mol)	Chemical Structure
2-Butenoic acid, 2-cyano-3-methyl- , ethyl ester	153,1784	
Benzoic acid, 2-benzoyl-, methyl ester	240,2540	

Figure 6 illustrates that the residue concentration in the dose 1 appeared lower than in the dose 2, except in the group C. The greater temperature provided an increase in enzyme activity [29], thus, it was shortening of the catalysis reaction time of substrate into products.

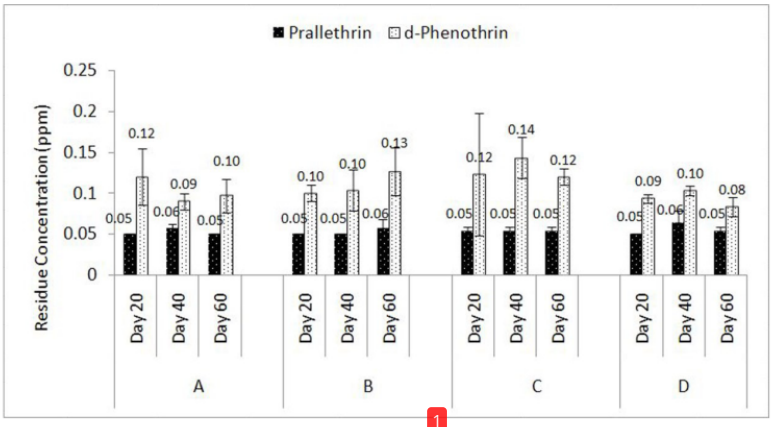


Figure 6. Residue Concentration in The Mouse Liver. Data were presented as Means ± Standard Deviation (SD).

This finding was confirmed by analysis of variance that there was significant effect between temperature variation and prallethrin residue (p-value = 0.025), however, it had no significant effects on *d*-phenothrin residue (p-value = 0,201). The simultaneous interaction of doses and temperature provided significant effects into d-phenothrin residue (p-value = 0.000), nevertheless, it did not occur in the prallethrin residue. In this research, the physicochemical aspects of prallethrin dan d-phenothrin comprised great influences into hydrolysis ability and stereoselectivity preferences in the biotransformation process by Ces1 towards specific isomers. Furthermore, the photolysis characteristics of *d*-phenothrin that easily degraded by ultraviolet delivered an enormous impact on the fluctuation concentration of *d*-phenothrin residue.

Discussion

Animal Body Weight

BW was, in fact, a complex indicator of tested animals that was greatly impacted by several factors among others. The welfare factors of tested animals (housing, husbandry, domination between animals, as well as the influence of environmental factors) [30–33], and the aspects of treatment that were provided to the tested animal [34]. The individual effect of these factors into the level of mice stress, which ultimately affected BW, has been examined by previous researchers. The effects of both individual and group

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housing [30,31], animals' aggressivity with housemates [31,32], competition [35], and population density in the cage [33], tested animals that were paired in one room with rat [36], transportation [37], circadian rhythm [38], cage position on the selves [39], illumination [40], and dark light cycles [41]. In this research, it was found that several factors other than welfare aspects were provided in aspects of research treatment including the activity of inserting animals into the chamber and being fasted for four hours. Afterward, the xenobiotics exposures were carried out in which containing acetonitrile and active ingredients of MR as well. The moments of these factors simultaneously interacted, and the complex impacts would emerge into BW, which possibly created a different effect, compared with when they were analyzed in a single aspect. Giving evidence that the application of research variables had no substantial effects on BW adjustment.

Temperature and Relative Humidity (RH)

RH underwent a saturation point in the range value of 99.90% ± 0% in all groups and occurred a constant from the beginning to the end of observation. Owen *et al*. [10] investigated that high RH enhanced the adhesion force of particles, which eventually would improve droplet condensation thereby changing particle distribution. The liquid molecules of elevated RH were adsorbed by particle surface and filled capillary space that ultimately inhabited in the contact area, thus, it improved particle adhesion. When small-size aerosol particles were deposited on a solid surface, these particles were attached to the contact area due to adhesion force [7]. These results were emphasized by statistical analysis of variance that illustrates no major differences in temperature in the chamber on all day measurements (p-value = 0,923). There was evidence to suggest that the exposure temperature in the chamber was similar from the beginning to the end of the observation.

Mass Concentrations of Particulate Matters

PMs are a highly heterogeneous complex mixture that was modified differently with respect to time and space. Kelly and Fussel [42] explained that PMs included many diverse chemical components and physical characteristics that many of which have been reported as potential contributors to toxicity.

Figure 4 illustrates that the group of C and D in a greater temperature relatively illustrate greater mass concentration compared with the A and B groups (groups with lower temperature) within the same dose. Giving evidence that the temperature increases in groups C and D were accompanied by the increase in particle size which ultimately affects mass particles in the same dose. Temperature played an important role in particle growth in which the increase in temperature would be followed by the enhancement of PM size. The PM that was smaller than the critical diameter would be evaporated including the mass contained within, thus, they contributed to the development of larger PM [10].

The mass of particles greater than $PM_{2.5}$ appeared containing a larger mass concentration than particles smaller than PM_1, and this pattern was quite similar in all groups. The ultrafine particles (≤ 1 μm) had a slight contribution to PM mass as described by Kelly and Fussell [42]. The mass of PM_1 was particulate mass with an aerodynamic diameter of ≤ 1 μm, the same definition was also applied into $PM_{2.5}$, PM_4, PM_7, and PM_{10}. While the definition of TSP was the total dry mass suspended solid in all measured particle size range. It happened since the operational principle of PM measuring devices was sucking air with a certain volume passed through a filter with a particular pore diameter. The PM mass concentration was obtained by measuring the mass of PM filtered divided by air volume, thus, the larger PM size would be enhanced the PM range included within. Therefore, the mass concentration of PM presented in Figure 4a illustrates that PM_{10} comprised the largest mass concentration compared with other PMs. The PM_{10} represented 97% - 100% of TSP on the same-day measurement, which was a very reasonable finding.

This finding was affirmed by ANOVA analysis that there was a significant effect of doses and temperatures into PM mass concentration (p-value = 0.017). This was also valid evidence that doses and temperature in simultaneous interaction provided a significant effect on particle size that eventually contained an important role in the mass concentration of PM.

Number Concentrations of Particulate Matters

The largest number concentration in the breathing zone was ultrafine PMs (≤ 1000 nm) which were inclined in Brownian motion. Thus, they undertook particle diffusion and tended to fill gradient concentration in the chamber including in the breathing zone. Instead, these PMs retained no significant

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contribution to mass particles, despite that, they are the largest number concentration and having a large surface area [43], providing an explanation of why an increase in number concentration did not accompany an increase in mass concentration. The characteristics of ultrafine particles produced the behavior of float flying in ambient air including in the breathing zone, therefore they had a great chance were inhaled by mice. Despite the fact that the particles > 1 µm significantly contributed to mass particles which were affected by gravity and inertia and thus tended to deposit at the bottom or surface wall of the chamber, so they had a small possibility to be inhaled by mice. The particles > 1 µm were affected by gravity and inertia forces [44] and thus tended to deposit in the bottom or surface walls of a chamber, while PM measurements were conducted in the mice breathing zone (± 7 cm from the bottom chamber) to represent inhaled PM concentration in mice. These varying forces might explain why PMs of a size of ≤ 1 µm was very abundant, while the size of 5 µm were almost zero, and those of size 10 µm were not measurable in the breathing zone. Kulkarni *et al*. [7] investigated particles of various sizes in terms of how they behave differently, determined by the application of physical laws. The ultrafine particles were arranged due to Brownian motion, while the large particles (micrometers) were influenced by gravitation and inertia forces. Gravitation force was proportional to mass particles and gravitational acceleration. The gravitational pull depended on the difference in particle density and surrounding media.

The temperature variation (Figure 2) played an important role in determining particle size [10,45] due to an effect on evaporation. Evaporation would occur when ambient air partial pressure was less than the vapor pressure saturation ($p_\infty < p_s$) that minimizes particle size. Saturated air containing water vapor in the chamber of 99.90% improved the adhesion force of particles. Owen *et al*. [10] investigated adhesion forces resulting from particle and surface properties, interface geometry, and condensed gas constituents. Once small aerosol particles were deposited on a solid surface, they adhered to the contact area due to these forces. The adhesive force could be increased by particle electrostatic charge, but high humidity could counteract this effect; thus, it improved the condensation of droplets which changed the size distribution of the particles. Condensation led to the growth of other particles' critical diameter that was used to determine the manner in which particles would increase in size. This particle diameter formed was dependent on vapor pressure. Particles smaller than the critical diameter would evaporate including their mass becoming available to aid in the growth of the larger particles [10]. In high humidity, liquid molecules were adsorbed onto the particles' surface and finally filled the capillary space and around the contact area [7].

ANOVA analysis confirmed that there was no significant effect of variable interaction on the number concentration of PM (p-value = 0.431). It was suspected that the number concentration detected was out of the detection limit of the PM device. Therefore, the number concentration did not provide an actual number of ultrafine PM in the chamber that was abundantly generated by bubble producing process.

Carboxylesterase (CE) Concentration

Carboxylesterase (CE) in mammals participated in the hydrolysis of ester, thioester, amide compounds containing xenobiotics and endogenous compounds [14] or in other words CE was essential in detoxication process of xenobiotics. The different concentration of prallethrin and *d*-phenothrin was relatively minor, thus, it provided a minor effect of an increase or decrease in carboxylesterase concentration as well. It was related to the activity rate of Ces1 to catalyze prallethrin and *d*-phenothrin isomers and their isomeric stereoselectivity preferences [46].

ANOVA analysis illustrates the significant effects of dose and temperature interaction into CE concentration (p-value = 0,001). Groups with temperature variations of 31°C (C and D) indicate CE concentration greater than the temperature of 25°C (A and B). Giving evidence that the temperature rises would increase enzyme activity and the denaturation process. Baket *et al.*, [47] investigated that the increase of *Ces*1 expression along with enzyme activity on the 1,4-bis-[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and PXR ligand pregnenolone-16a-carbonitrile (PCN) administration. These findings were supported by an analysis of variance that temperature played an important effect on CE concentration (p-value = 0.000). It was implied to suggest that a similar dose provided diverse consequences of CE concentration in the modified temperature of environmental conditions.

The Metabolite and Residue of Prallethrin and *d*-Phenothrin Mixture

Biotransformation process of pyrethroid including prallethrin and *d*-phenothrin was dominated by the hydrolysis process of ester, thioester, and amide compounds. This process produced discarded parent chemical (or also called by the residue of parent chemical) and intermediate compounds as well (or also

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called by metabolite) that resulted from breaking down the covalent bonds. This separation was conducted to obtain the compound with a smaller molecular weight compared to the parent chemical, thus, they were simply excreted through urine.

Benzoic acid, 2-benzoyl-, methyl ester was an intermediate compound or also called the metabolite of prallethrin and d-phenothrin mixture. Even though, these findings were dissimilar with the previous research that generally found 3-phenoxybenzyl alcohol or 3-phenoxy benzaldehyde (PBA) as it has been examined by Thiphom et al. [48]. Thiphom et al. [48] investigated cyfluthrin, permethrin, and cypermethrin exposures. While 2-butenoic acid, 2-cyano-3-methyl-, ethyl ester was not believed to be a metabolite of prallethrin and d-phenothrin mixture, other than, the metabolite of acetonitrile that was employed as a solvent. prallethrin and d-phenothrin were typed I of pyrethroid that contained no cyano group [49], thus, the cyano group of this metabolite was suspected derived from acetonitrile which was metabolized into inorganic cyanide. The previous study examined the reaction of acetonitrile with P-450 produced cyanohydrins that were composed by catalase to release cyanide [50–52].

The residue of the parent chemical and metabolite in the liver could be employed as a parameter of the biotransformation process of prallethrin and *d*-phenothrin. Once the enzyme was appropriately performed, the hydrolysis process was carried out according to the enzyme activity rate and generated the metabolite which would be excreted through urine and exhaled breath (both as a CO_2 and/or as a parent chemical). Otherwise, if the enzyme performed was obstructed (for example due to the existence of an inhibitor), hence there was a decreased concentration of exhaled CO_2. Consequently, the residue and/or metabolite excreted through urine and/or there was accumulation occurred in specific organs/tissues which were suspected to exacerbate health conditions. In addition, the research with a continuous exposure system that was executed by providing substrate supply in succession beyond the enzyme degradation ability of xenobiotics according to co-enzyme activity rate (nmol/min-mg protein), also played a role in residue and/or metabolite accumulation.

Prallethrin and *d*-phenothrin are members of the pyrethroid family and are analyzed as particulate matters (PMs) with respect to inhalation exposure with a variety of doses and temperatures. The results illustrate a polydisperse distribution of PMs, with the major contribution being ultrafine particles in terms of concentration of the largest (particles/L) of diameter 0.3 µm, followed by 0.5 µm, 1 µm, and 5 µm. Even though these PMs are the largest generated, however, they have no crucial contribution to mass PM. The simultaneous interaction of doses and temperatures provides significant effects into mass PM, nevertheless, it does not occur in the number concentration of PM. The carboxylesterase (CE) concentration was an adaptive response of the mouse towards an increase or decrease of substrates (doses variation) and environmental conditions (temperature variation). The simultaneous interaction between both of them generates important effects on CE concentration. CE concentration was directly proportional to the increase or decrease of protein synthesis that illustrates the increase or decrease activity rate of Ces1 towards specific isomers of prallethrin and *d*-phenothrin. The simultaneous interaction between both doses and temperature variation also provides a significant effect on the residue concentration of *d*-phenothrin residue, however, it does not occur in the prallethrin residue. It is suspected due to physicochemical characteristics and the stereoselectivity preferences of Ces1 towards isomers of prallethrin and d-phenothrin. This research also finds benzoic acid, 2-benzoyl-, methyl ester that is believed as a metabolite of prallethrin and *d*-phenothrin mixture, that have been found yet in the previous studies. This study suggests that PMs generated from prallethrin and *d*-phenothrin mixtures contain ultrafine particles and giving evidence that they are hazardous in terms of inhalation exposure for living organisms according to CE concentration, the accumulation of residue and metabolite in the mouse liver. Further studies will be performed in order to characterize the specific activity and stereoselectivity of Ces1 towards a specific isomer of prallethrin and/or *d*-phenothrin that was suspected to influence both metabolite and residue resulted.

Declaration of Competing Interest

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

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Ethical Clearance

All procedures performed in studies involving animals were in accordance with the ethical standards of the Animal Care and Use Committee (ACUC) of the Veterinary Faculty of Airlangga University with certificate number 716-KE.

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