

Carboxylesterase Concentration in Mouse Exposed to Particulate Matters on Inhalation Exposure of Prallethrin and *d*-Phenothrin Mixture

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Abstract. CE enzyme applied critical hydrolysis of pyrethroid families such as prallethrin and *d*-phenothrin, this active ingredient was commonly contained in mosquito repellent (MR). The previous study found that MRs as particulate matters (PMs) were very hazardous for living organisms due to the characteristics of number and mass concentration. This study examined the CE concentration in *Mus musculus* exposed to PM on inhalation exposure of prallethrin and *d*-phenothrin mixture. The lower dose was a mixture of 0.000141 mg/L prallethrin and 0.104 mg/L *d*-phenothrin, while the higher dose was a mixture of 0.00141 mg/L prallethrin and 1.04 mg/L *d*-phenothrin. Prallethrin and *d*-phenothrin were dissolved in acetonitrile, then diluted several times to obtain the preferred concentration. The solution was inflated with air through a diffuser to generate PMs which inserted into the chamber contained mice. The experimental group was divided into three, namely: positive control (PC), and lower- and higher-dose treatment groups, with three replicates for each group. The results illustrate that lower and higher dose demonstrated major differences. The statistical analysis confirmed that CE concentration had significant differences between groups. The increase in pyrethroid concentration followed by the increase of CE concentration, It indicated that the increasing CE substrates would be followed by the increasing of protein synthesis including CE. PMs in terms of number concentration of the largest (particles/L) is 0.3 μm , followed by 0.5 μm , 1 μm , and 5 μm . Approximately 99.86 % of the mass concentration the breathing zone is contributed by respirable particles (fine and ultrafine particles). Even if ultrafine particles are the largest number concentration, they have no significant contributions to the mass. A very abundant of fine and ultrafine particles affects they were beyond detection limit instruments, thus, they have no significant relationship with CE concentration, even though number concentration is more prominent than the mass concentration in the toxicological field, due to the high surface area of ultrafine particles.

Keywords: **Particulate matters; carboxylesterase; prallethrin; d-phenothrin; inhalation; pyrethroid.**

1 Introduction

The previous studies suggest that indoor particulate matter (PM) produces health disorders [1, 2]. It is essential to obtain seriously consideration since people spend most of their time (80%) indoors [3]. Indoor human activity which significantly generates PMs in tropical countries is the use of mosquito repellents (MRs) either in the form of mats, sprays or coils [4, 5].

PM is a heterogeneous complex mixture between solid and/or liquid [6] which size and chemical properties of PMs are important parameters to determine particle behavior [7] including the significant aspect relating to deposition particles in the lungs as well [6]. Owen *et al.* [8] studied PM in the aerodynamic diameter range of > 30 μm have a low probability of penetrating nasal passages, and Phalen *et al.* [9] investigate the particles with an aerodynamic diameter of 5 – 10 μm deposited in the nose and pharyngeal passages favor

rapid and sharp airflow, thus, if the particle size is reduced from micrometer to nanometer range, increasing toxicity appears due to the ability to penetrate respiratory system [9] and increase in particle surface area [10].

Carboxylesterase (CE) plays a crucial role in metabolism, detoxification, and elimination of endogenous and exogenous ester [11] as well as pyrethroid families like prallethrin and *d*-phenothrin. These enzymes can hydrolyze a wide variety of ester, amide, and thioester substrates [12]. Nevertheless, many CEs demonstrate stereo-/enantioselective hydrolysis toward certain substrates [13]. These enzymes produce low until moderate toxicity of pyrethroid biodegradation into human and mammalian liver on oral exposure [14]. Other researchers investigate that high-level exposure of pyrethroid in the long term is significant to generate health disorders [15, 16].

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Recently, mosquito repellents are employed to reduce the mosquito population especially in tropical countries, which commonly contain two or three compounds to produce expected outcomes. However, a study examining the MR compounds mixtures effects as a particulate matter exposure on carboxylesterase concentration has yet to be studied, even though MR as a single compound has been investigated by researchers. Matsunaga *et al.* [17] examined prallethrin application in an indoor environment, while Okuno *et al.* [18] investigated the insecticidal activity of *d*-phenothrin. Thus, it is very important to investigate CE concentration on the metabolism of prallethrin and *d*-phenothrin mixture in inhalation exposure.

This study examines the CE concentration in inhalation exposure of prallethrin and *d*-phenothrin mixture in the mouse.

2 Materials and Methods

2.1 Chemicals and Reagents

Prallethrin and *d*-phenothrin were obtained from Sigma Aldrich with a catalog number of prallethrin 32917 and *d*-phenothrin 36193. The doses of prallethrin and *d*-phenothrin were the lower and higher doses. The lower dose was a mixture of 0.0001 mg/L prallethrin and 0.104 mg/l *d*-phenothrin, while the higher dose was a mixture of 0.001 mg/l prallethrin and 1.04 mg/l *d*-phenothrin. The lower dose was determined from the NOAEL value of prallethrin and *d*-phenothrin of 28 days' exposure [19], while the higher one was ten times larger than the lower dose. These active ingredients were diluted several times to obtain the preferred concentration. Doses were provided for each mouse.

2.2 Experimental Groups of Inhalation Exposure

Groups were divided into three, namely: positive control (PC) and treatment (lower and higher dose) groups (Fig. 1). The PC was the experiment with exposure to solvent only, whereas lower-dose treatment groups were the experiment exposed to a mixture of 0.0001 mg/L prallethrin and 0.104 mg/l *d*-phenothrin exposures and higher-dose treatment groups involved a mixture of 0.001 mg/l prallethrin and 1.04 mg/l *d*-phenothrin. There were three chambers for replication for each group, and each replicate contained three mice.

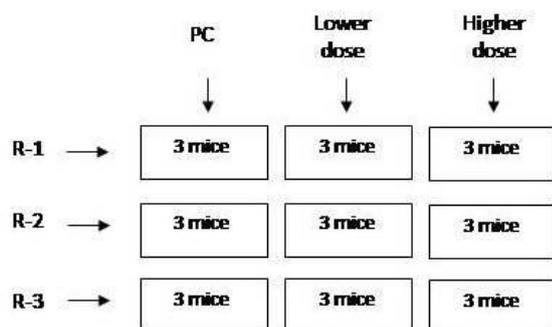


Fig. 1. Experimental Group Illustration of PM Exposure

2.3 Technique of Inhalation Exposure

Prallethrin and *d*-phenothrin were dissolved in acetonitrile and then diluted several times in distilled water [20], that used for inhalation exposure to 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, discharged at 4 L per minute into the solution [21] to produce PMs. The PMs 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' observation of prallethrin and *d*-phenothrin mixture. The experiment was executed at temperatures of $31.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and RH of $94\% \pm 2.5\%$ in a continuous system chamber. The exposure technique described was already calibrated.

2.4 Measurement of Carboxylesterase Concentration in Mouse Liver

CE concentration was determined using Mouse Carboxylesterase ELISA kit from Bioassay Laboratory manufacturing, Shanghai-China. The standard curve was depicted with Curve-Expert Software with five gradient concentration 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 employed to determine the concentration of CE concentration.

2.5 Measurement of Particulate Matters, Temperature and Relative Humidity

PMs were measured at the third hour of exposures 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. Temperature and relative humidity (RH) were evaluated using a Son off TH Sensor AM2301 from ITEAD (China), that measured temperature and RH every five minutes for four-hour exposures during 60 days of observation. Readings were monitored using an android system and automatically kept in the data logger. All equipment and apparatus used were already calibrated.

2.6 Measurement of Mouse Body Weight

The weight gain 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.

2.7 Animal husbandry and maintenance

Six-week-old male BALB/c mice were obtained from the Airlangga University pharmacology laboratory 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 mice were housed together with 12 mice 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 observation. The study was approved by the Animal Care and Use Committee (ACUC) of the Veterinary Faculty of Airlangga University with certificate number 716-KE.

2.8 Statistical Analysis

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.

3 Results and Discussions

3.1 Mouse Body Weight

Fig. 3 illustrates that the mean body weight of negative control increased significantly, with the largest weight on the 40th day even up to 45.4 g ± 3.6 g, subsequently on the 60th day this reduced to 37.1 g ± 4.7 g, whereas weight gain patterns of positive control and treatment groups (lower and higher dose) were similar. There were no significant increases in body weight on days 20, 40 and 60, even likely to be constant.

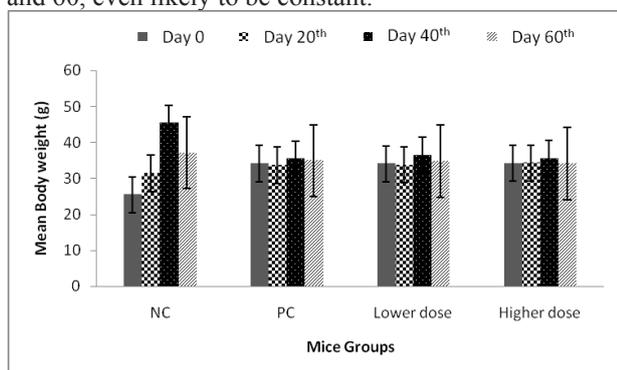


Fig.2. Body Weight of Mice. Data were presented as means ± standard deviation (SD).

The influence of PMs exposures (with respect to all diameter particles) against body weight was statistically significantly different in body weight between negative and positive controls and lower-and higher-dose treatment groups (p-value = 0.021); however, there were no significant differences between positive control and treatment (lower and higher dose) groups (p-value = 0.402). The body weight gain by age indicates healthy mice with excellent growth and development at the acclimatization stage (data not shown). This result presented evidence that the mice were in a healthy

condition prior to the treatment being carried out. The body weight was a growth indicator that was simultaneous consequence of many factors' interaction. At the time of acclimatization, factors affecting weight included the social environment of mice including caging, aggressiveness, contact with the rat in the animal room [22–26], husbandry [27], new environment, duration and mode of transportation, noise, vibration and reduction of food and drink availability [28]. Interactions among factors that were affected were increasingly complex when treatment was executed, due to the process and materials employed for treatment which affected body weight as well [29]. Thus, the result findings no significant differences between the negative control and treatment (lower and higher dose) groups were reasonable.

3.2 Carboxylesterase Concentration

Carboxylesterase (CE) was very abundant both human and rodent liver [29]. The recent studies had identified that CE was responsible for biotransformation reaction especially CE-1 both in humans (CES1) and rodents (Ces-1) [30]. The substrate specificities of pure human and rodent CE proteins toward pyrethroids reveal substantial differences. Several type II pyrethroids, which are distinguished from type I pyrethroid compounds by the presence of a cyano functionality on the alcohol moiety of the pyrethroid, were markedly faster rates than by a rat CES1 ortholog [29], thus, the rodent model was more suitable in pyrethroid metabolism.

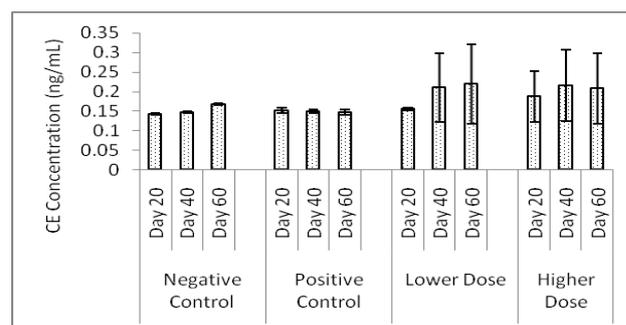


Fig. 3. CE Concentration in Mice. Data were presented as means ± standard deviation (SD).

CE concentration illustrates significant differences between day 20th, 40th and 60th (p-value = 0.000) in all groups. There was an increased CE concentration in a linear manner along with the numbers of days in the negative control (Fig. 3), while in positive control showed a relative constant. The lower and higher dose demonstrated major differences. The lower dose was quite linear compared with the negative and positive control, while the higher dose reached steady state at day 40th and 60th.

In the reaction between enzyme and substrates, the catalytic site of the enzyme contained shaped-like-substrates, thus, it was very specific to the appropriate substrate. ELISA analyzed according to the antigen-specific monoclonal antibodies production, so, the only

substrate with specific antigen would be bound by antibodies, while non-specific substrate would be removed at the washout step. Positive control exposures only contained acetonitrile which was not a specific substrate of CE, thus, it was a reason why the CE concentration in the positive control was relatively constant.

The statistical analysis confirmed that CE concentration had significant differences between groups. The higher dose illustrates the elevated CE concentration compared to the lower dose, giving evidence that the substrates enlarged (mixture compound) would be followed by the increase of CE concentration, due to the increase of protein synthesis. Godin *et al.* [20] clearly demonstrate the species difference in the activities of rat hydrolase A and hCE-1 that was unique for deltamethrin when compared with the general similarity in hydrolytic rates observed with the other pyrethroids. Hosokawa *et al.* [11] found that the concentration of CE isozymes varies with life stage, individuals, sex, and species resulting in differential susceptibility to the same pyrethroid, thus, the high value of standard deviation in lower and higher dose was reasonable.

3.3 Temperature and Relative Humidity

Temperature and relative humidity (RH) played an important role in determining the behavior of PM.

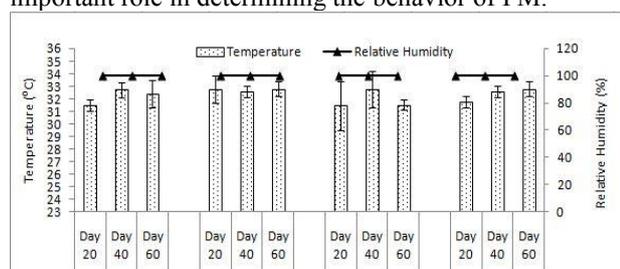


Fig. 4. Temperature and Relative Humidity in the Breathing Zone. Data are presented as means \pm standard deviation (SD).

The negative control group was the mice without exposure at all by supplying air that inserted into the chamber, thus, the chamber just contained air supply from the outside, exhaled respiration and heat that was resulted from tested animal metabolism, therefore, the increasing temperature in the negative control group was reasonable, moreover, the temperature outside provided an effects to inside.

The positive control group exposed with acetonitrile only (pyrethroid solvent) which dissolved in distilled water. The solution aerosolized and inserted into the chamber, thus, the temperature in the positive control group was a relative constant due to saturated relative humidity. The temperature of the lower dose was 31.5 °C at day 20 and elevated at day 40 of 32.7°C, then, they declined on day 60 of 31.5 °C. The higher dose temperature illustrates the linear enlargement on day 20 until day 60. The temperature played a crucial role in particle growth. The increasing of temperature would be improved particle size. Particle smaller than the critical diameter would evaporate including mass particle inside [8].

3.4 Mass and Number Concentration of Particulate Matter

The highest concentration was PM₁₀ followed by PM₄, PM₇, PM_{2.5} and PM₁ in PC, lower and higher dose, and PM₁₀ was not measurable. Fine particles ($\leq 2.5 \mu\text{m}$) even comprised the highest number concentration (Fig. 5b), they had a minor contribution to PM mass, even though there was a high number concentration (Fig. 5b).

There were a significant difference in PM mass concentration ($\mu\text{g}/\text{m}^3$) between PC, higher and lower dose groups (Fig. 5a), and it was no significant differences of PM mass concentrations ($\mu\text{g}/\text{m}^3$) in the same particle size of all groups in PM₁ (p-value = 0.696). However, there were a significant differences of PM mass concentrations ($\mu\text{g}/\text{m}^3$) in size of PM_{2.5} (p-value = 0.009), PM₄ (p-value = 0.000), PM₇ (p-value = 0.000), PM₁₀ (p-value = 0.000) and TSP (p-value = 0.000).

PM number concentration in breathing zone (Fig. 5b) illustrates a polydispersed distribution with the major contribution being ultrafine PMs (diameter of $\leq 1 \mu\text{m}$), with the largest concentration in a diameter of 0.3 μm , followed by 0.5 μm , 1 μm , and 5 μm . The same pattern 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. The largest number concentration in the breathing zone was ultrafine particles ($\leq 1000 \text{ nm}$) which were tended in Brownian motion. Thus, they carried out particle diffusion and inclined to fill gradient concentration in the chamber including in the breathing zone [6].

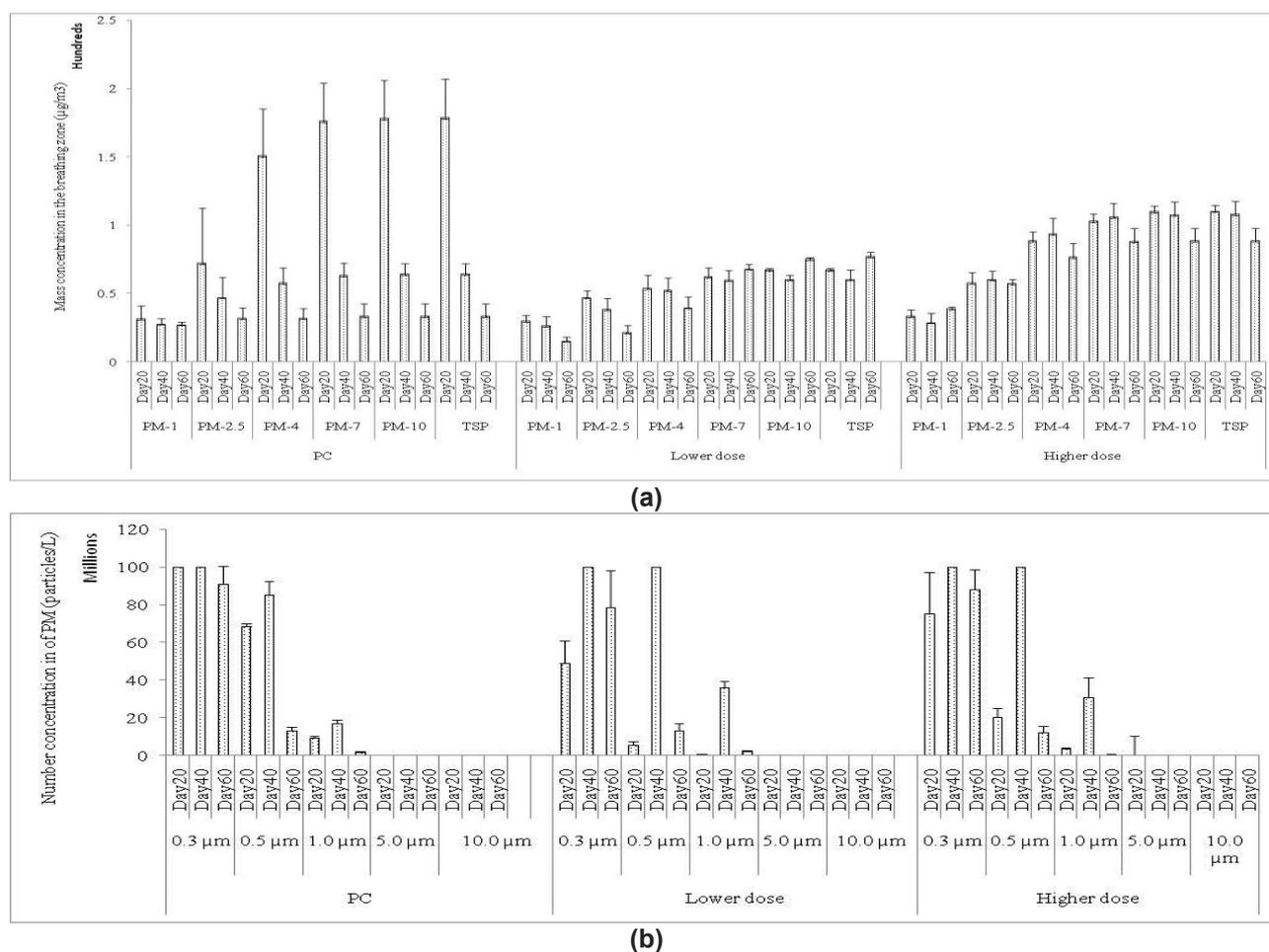


Fig.5. Mass Concentration (a) and Number Concentration (b) of PM at different days and groups in the breathing zone. Data are presented as means \pm standard deviation (SD).

The larger particles diffused more slowly than the smaller ones [6]. The particles with the size of > 1000 nm were influenced by the gravity and inertia forces [31], thus, had a tendency to deposit in the bottom and/or surface walls of a chamber, while PM measurements were executed in the mice breathing zone (± 7 cm from the bottom chamber) to represent inhaled PM concentration in mice. These varying forces provided evidence why PMs of a size of $\leq 1 \mu\text{m}$ were very abundant, a size of $5 \mu\text{m}$ were almost zero and those of size $10 \mu\text{m}$ were not measurable in the breathing zone. Kulkarni *et al.* [6] investigated particles of various sizes in terms of how they behave differently, which determined by the application of physical laws. Gravitation force was proportional to mass particles and gravitational acceleration. The gravitational drag was depended on the difference of particle density and the surrounding media. The buoyancy effects could be neglected for compact particles for the particle in the air.

The difference in temperature and RH (Fig. 4) played a critical role in determining particle size [8, 32]. Saturated air containing water vapor in the chamber of 99.90% increased the adhesion force of particles. Owen *et al.* [8] investigated adhesion forces resulting from particle and surface properties, interface geometry and condensed gas constituents. The small aerosol particles were deposited on a solid surface, they would adhere to

the contact area due to these forces. The adhesive force could be enlarged by particle electrostatic charge, other than the high humidity could counteract these effects; thus, it improved the condensation of droplets which would change the size distribution of the particles. Condensation led to the growth of other particles' critical diameter that was applied 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 be evaporated with their mass becoming available to aid in the growth of the larger particles [8]. In high humidity, liquid molecules were adsorbed onto the particles' surface and filled the capillary space at the end and around the contact area [6].

PMs in aerosols which generated from the bubbles-producing process were ultrafine PM [33]. The PM size which was formed was influenced by the number of suspended particles and the viscosity of solution that affected later on surface tension. Thus, this was a reason why these three-groups had different doses, indicating a significant difference in the number concentration of PM.

3.5 Relationship between Particulate Matter Exposures and Carboxylesterase Concentration

The ANOVA analysis showed no significant relationship between CE concentration and both of particulate matter concentration, mass (p-value= 0.389) and number concentration (p-value =. 0.820). The aerosol generated from bubble production produced a very abundant fine and ultrafine PMs [33]. They had no crucial effects on mass concentration, even though comprised the largest proportion of all PMs generated. This results provided evidence of why the mass concentration has no significant correlation with CE concentration. A very plentiful of fine and ultrafine PMs generated were beyond the detection limit of instruments (an Aerocet 531S Particle Mass Profiler and Counter from Met One Instrument Inc (USA)), thus, these result findings were reasons why the number concentration had no significant relationship to CE concentration. Actually, the fine and ultrafine PMs were small enough to reach the alveolar region, and approximately 100% of the number of particles could reach the alveolar region, so the number concentration of PMs should be “more prominent” than the mass concentration in the toxicological field, due to the high surface area of ultrafine particles.

4 Conclusion

Prallethrin and *d*-phenothrin are members of the pyrethroid family and are analyzed as particulate matters (PMs) with respect to inhalation exposure. CE enzyme applies critical hydrolysis of pyrethroid family. The increase of doses will be followed by the enlargement of CE concentration. PMs in terms of number concentration of the largest (particles/L) is 0.3 μm , followed by 0.5 μm , 1 μm , and 5 μm . Approximately 99.86 % of the mass concentration the breathing zone is contributed by respirable particles (fine and ultrafine particles). Even if ultrafine particles are the largest number concentration, they have no significant contributions to the mass. A very abundant of fine and ultrafine particles affects they were beyond of detection limit instruments, thus, they have no significant relationship with CE concentration, even though number concentration is more essential than the mass concentration in the toxicological field, due to the high surface area of ultrafine particles.

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