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Temperature and pH Stability Profiles of *ortho* and *para* DEET

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ABSTRACT – DEET, *N,N'*-diethyl-*m*-toluamide, is the most commonly used mosquito repellent. However, it can easily permeate through skin leading to toxic effects. A recent study showed that the *ortho* analogue of DEET showed enhanced repellency with reduced permeation compared to the commercially used *meta* analogue. Thus, in order to understand the differences in properties and effectiveness among the *m*-, *o*- and *p*-analogues of DEET, an HPLC-UV method was developed for separately analyzing the three analogues. Moreover, stability profiles at temperatures ranging from 30°C to 70°C as well as pH ranging from pH 3 to pH 9 have been determined. All three analogues were stable with no degradation observed during the 5 day period. *o*-DEET therefore could be further developed into a safer and more effective mosquito repellent.

Key words – DEET, Temperature stability, pH stability, Mosquito repellency

m-DEET is known as the golden standard of mosquito repellents for being highly effective, stable and relatively safe (Katz et al., 2008). In most cases it is directly applied to the human skin protecting against tick-borne diseases (Jensennius et al., 2005) and mosquito-mediated diseases including dengue fever, West Nile virus and Eastern Equine Encephalitis (Gubler, 2001). However, information about the mechanism of action of DEET is still controversial. Studies report that DEET may block behavior of mosquitoes by inhibiting chemo-receptors on the mosquito antennae that are stimulated by lactic acid (Dogan et al., 1999) whereas other studies refute this phenomenon (Syed and Leal, 2008). Nevertheless, *m*-DEET exhibits mosquito repellency as shown in the complete protection time (CPT) ranging from 203 to 756 minutes, varying with factors including climatic effects, mosquito species and physical activities (Barnard and Xue, 2004; Bidlingmayer, 1994; Bernier et al., 2000).

The search for safer and more effective repellents continues to progress, however, due to the toxicities reported of DEET (Schofield et al., 2007). Possible substitutes to DEET that have been reported so far include synthetically prepared Picaridin, IR3535 and Permethrin or lemon eucalyptus or citronella which are natural products (Katz et al., 2008). Unfortunately, none of these alternatives seem to exhibit properties that could be more advantageous to the current DEET products.

Current research for developing repellents make use of systematic bioassays or computer based tools which include a QSAR study of *N*-acyl-piperidines (Katritzky et al., 2008) and a genetic trial of odorant-binding protein manipulation (Li et al., 2008). One of the shortcomings of the mosquito repellent investigation is the use of human subjects or living vertebrates in the bioassays (Dogan et al., 1999). However, recently, an efficient *in vitro* bioassay system has been established where blood substitute and collagen membrane were used instead of human blood and skin (Jahn et. al., 2010). The artificial blood substitute was a slight modification of that used by Kogan for an *in vitro* feeding assay for maintenance of mosquito colonies (Corazza et al., 2005). Unlike Kogan who utilized stretched parafilm as a skin substitute, Jahn made use of a collagen type membrane (Ditzen et al., 2008) since the parafilm alone could not mimic the physiological skin sufficiently. Moreover, in Jahn's study, skin permeation profiles of DEET and its analogues were examined in correlation to the mosquito repellency, expressed as CPT. Among the DEET analogues tested, compared to the commercially used *meta* DEET analogue, *ortho* DEET revealed enhanced repellency with reduced skin permeation rate, implying that toxicity due to skin penetration would be lessened (Jahn et al., 2010).

To estimate the applicability of the *ortho* DEET analogue, a separation method for the three DEET isomers needed to be established. Because of the similar physicochemical characteristics of the three isomers as revealed in the capacity factors

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determined by HPLC, being able to separately analyze the *ortho* isomer from the *meta* isomer has been a challenge (Jahn et al., 2010). In addition, the physical stability of the *ortho* analogue needs to be established in order to develop the appropriate formulation.

Therefore, in continuation of our search for a safer and more effective mosquito repellent, we herein report on the pH and temperature stability of *ortho*-DEET, together with an improved HPLC analysis method.

Materials and Methods

Chemicals and reagents

meta-DEET was purchased as DEET(97%) from Aldrich Chemical Co.(St. Louis, Mo.,USA). The *ortho* and *para* isomers were synthesized as reported by Jahn et al. (2010). The structures are shown in Figure 1. Double-distilled water was acquired using a Millipore system. HPLC-grade methanol, water and acetonitrile were purchased from J. T. Baker, USA. Sodium acetate and potassium phosphate for the buffer preparation were obtained from Sigma-Aldrich (St. Louis, Mo., USA) and were of analytical grade or better. Phosphoric acid (85%, KANTO chemicals) and potassium hydroxide (85%, TEDIA, Fairfield, OH, USA) were used for the pH adjustment of the phosphate buffer while sodium hydroxide and glacial acetic acid (Yakuri Pure Chemicals Co., Ltd., Kyoto, Japan) were used for the acetate buffer. All other reagents were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and were of synthetic grade or better.

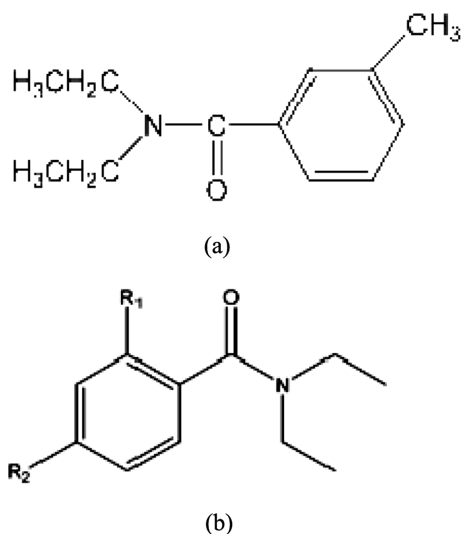

Figure 1. Chemical Structures of DEET analogues: (a) structure of *meta*-DEET which is the analogue used in commercial products; (b) *ortho* DEET where R_1 = CH_3, R_2 = H and *para* DEET where R_1=H, R_2= CH_3.

HPLC analysis

The liquid chromatographic system consisted of SHIMADZU Solvent Delivery Module LC-10AD (VP) Separation Module and a SHIMADZU UV-Vis Detector SPD-10A(VP) lambda absorbance detector. RP-18 Lichro-cart® 125 Lichrosphere 100 (5 μm, 150 × 4 mm) was purchased from MERCK (Darmstadt, Germany) while the C18 reversed phase column (U6120, S-5 μm, 4.6 mm I.D. 250 × 5 mm) was purchased from SHISEIDO (Japan). A SHISEIDO Capcell guard column was also used. The mobile phase consisted of 0.5 M acetate buffers or 0.3 M phosphate buffers of varying pHs and methanol or acetonitrile and water at varying constitutions. They were filtered through RC membrane filters of 47 mm, 0.45 μm size (Sartorius, Goettingen, Germany) and degassed in a Branson 1210 Heat Sonic Bath (Model 1210R-DTH). The Corning pH meter 440 was used for pH measurements of buffer solutions.

Separation of DEET isomers by HPLC

Separation of the three analogues of DEET was pursued by finding optimum HPLC conditions by varying the length, inner diameter and packaging of the columns, altering the mobile phase compositions and flow rates. A combination of methanol, water and/or acetonitrile was tested for the mobile phase while the flow rate was varied from 0.6 to 0.9 ml/min. The HPLC condition is as described above with detection at 220 nm based on a wavelength scan analysis by UV (data not shown). The injection volume was 25 μL. Resolution factors were calculated using the formula:

$$R = (t_{R2} - t_{R1}) / (w_1 + w_2)$$

where t_R is the retention time and w the peak width.

Sample preparation for stability tests

For pH stability tests of the *meta*-, *ortho*- and *para*- DEET analogues, buffers of pH 3, 5, 7, and 9 were made. The buffers of pH 3 and 5 were maintained using a 0.5 M acetate buffer and those of 7 and 9 were maintained using a 0.3 M phosphate buffer, respectively.

All samples were prepared in glass ampoules of which the final volume was 10 mL. Adequate concentrations of DEETs were dissolved in methanol after which the solvent was blown away with nitrogen and filled up to the appropriate volume using buffers of different pHs. For temperature stability studies, the Sanyo drying over (maintained at 70°C) and Hanbaek incubator (HB 201 SF, maintained at 30°C and 50°C) were used.

Stability studies were conducted for 5 days. During this 5 day period, samples were removed daily and stored in a -20°C freezer prior to HPLC analysis. At a wavelength of 220 nm, a mobile phase composed of methanol and water (60:40, v/v) was used at a flow rate of 0.8 ml/min. RP-18 Lichro-cart® 125 LiChrosopher 100 (5 μm, 150 × 4 mm, Merck, Darmstadt, Germany) was used as the stationary phase.

Statistical analysis

Statistical analysis was performed using a Student t-test at the $p<0.05$ level. All experiments in the study were repeated at least three times and all data were presented as the mean ± standard deviation.

In vitro rat skin permeation and deposition study

The experimental protocols involving animal study were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University. The permission number of animal experiment is "SNU-200909-33." The rat used for the preparation of skin (Sprague Dawley, male, 220-250 g) were obtained from OrientBio Co. (Seongnam, Korea). The animals had free access to food and water until used for experiments, and were sacrificed in a CO_2 chamber right before the experiments. The dorsal hairs were removed with a clipper and full-thickness skin (about 10 cm^2) was surgically removed from each rat. The skin specimen was cut into appropriate sizes after carefully removing subcutaneous fat and washing with normal saline. The *in vitro* skin permeation across the rat skin was conducted with Keshary-Chien diffusion cells at 37°C. Freshly excised rat skin was mounted between the donor and receptor cell (stratum corneum side facing the donor). The area of diffusion for all *in vitro* experiments was 2.01 cm^2. The receptor cells, which faced the dermis side, were filled with pH 7.4, 10 mM phosphate buffer solution (12 mL). At predetermined time intervals, 1ml of the receptor solution was withdrawn and refilled with the same volume of fresh receptor solution. Samples were kept in a freezer (-20°C) until analyzed by HPLC. For the skin deposition study, the skins were thoroughly washed with methanol, after which the surface was dried with a cotton swab and DEET content in the dermis skin layer was determined. The effective surface area was separated and minced with a surgical sterile scalpel then finally homogenized with methanol by using ultra turrax homogenizer at 16,000 rpm for 5 min (T25 Basic, Germany) on ice bath (4°C). The tissue suspension was centrifuged for 5 min at 3,000 × g, and then the supernatants were filtered and assayed by HPLC.

Results and Discussion

Separation of DEET analogues

The optimum condition for separating the three DEET analogues was best achieved using a reversed phase C18 column (U6120, S-5 m, 4.6 μm, ID., 250 × 5 mm) from SHISEIDO (data not shown). However, retention times at which the DEET analogues eluted were 34.34 min, 45.24 min and 46.78 min for the *ortho*, *meta*, and *para* analogues respectively, which was too long a run for repeated analyses. Thus to decrease the run time, a shorter C18 column of 125 mm was used even though dispersion occurred to a certain degree. Figure 2 shows the chromatograms where different mobile phase compositions

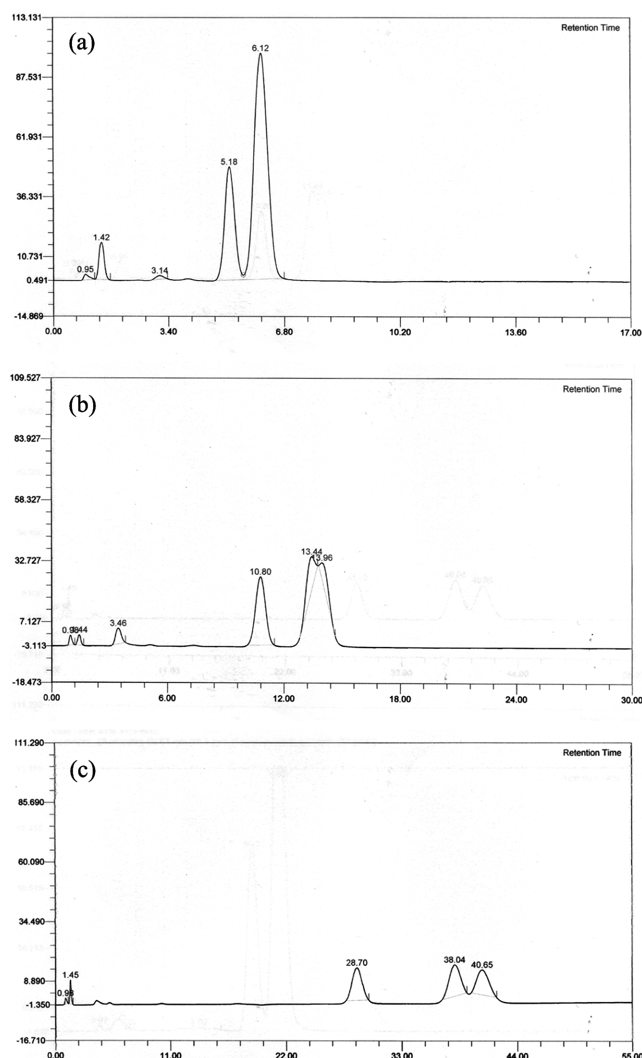

Figure 2. The influence of mobile phase composition on the separation of DEET analogues: (a) Methanol: water (6:4), (b) methanol: water (1:1), (c) methanol: water (4:6). All experiments were done using HPLC-UV (220 nm) system using RP-18 Lichrocart® 125 LiChrosopher 100 at a flow rate of 0.8 ml/min.

were used when analyses were conducted using this 125 mm column. The DEET isomers at all times eluted in the order of *ortho*-, *meta*- and *para*- analogues respectively. Moreover, the *ortho*-DEET at all times did not overlap with the other two peaks whereas resolution of the *meta* and *para* was always a challenge. The optimum separation condition was further ascertained by the stipulation and comparison of the resolution factors as shown in Table I. Resolution factors are calculated based on the difference in retention times divided by the sum of the widths of the two peaks as shown in the equation in the Materials and Methods section. The larger the value, the better separation was achieved. A resolution factor of 1.0 was achieved when using methanol: water (42:58, v/v) as mobile phase at a flow rate of 0.9 mL/min. However, satisfactory resolution (1.0 ± 0.3) was observed with methanol: water (42:58, v/v) at a flow rate of 0.8 mL/min. Figure 3 shows the relationship between resolution factor and polarity of the mobile phase (in % methanol). Although resolution was improved as the polarity of the mobile phase increased, the delay in retention time should be taken into consideration as well especially when routine repeated analysis should be conducted. Therefore, it was decided that for repeated analyses, conditions with lower resolution be utilized after which characterization of the analogue be made using the method where resolution is higher.

Table I. *Resolution factors (R) between meta and para DEET using different mobile phase compositions and varying flow rates with a 250 mm C18 column*

Mobile phase	Flow rate	R	rt *p*-DEET [min]
MeOH:water [50:50, v/v]	0.6 mL/min	0.90	44.12
	0.7 mL/min	0.77	31.87
	0.8 mL/min	0.76	27.69
MeOH:water [48:52, v/v]	0.6 mL/min	0.87	38.94
	0.7 mL/min	0.84	33.32
	0.8 mL/min	0.76	29.25
MeOH:water [46:54, v/v]	0.7 mL/min	0.85	38.93
	0.8 mL/min	0.81	34.01
MeOH:water [44:56, v/v]	0.8 mL/min	0.99	42.51
	0.9 mL/min	0.89	37.83
MeOH:water [42:58, v/v]	0.8 mL/min	1.03	52.54
	0.9 mL/min	1.00	45.78

rt: retention time

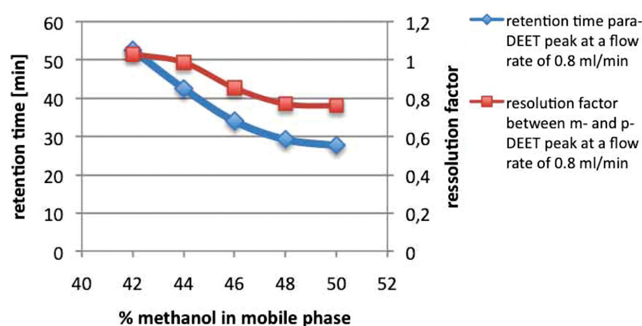

Figure 3. Resolution factor vs. polarity of mobile phase. As the percentage of methanol increased in the mobile phase, better resolution was observed although retention time was delayed.

Stability tests

The stability profiles revealed *ortho*- and *para*- DEET to be as stable as the commercially used *meta*-DEET. All three isomers were tested for temperature stability at 30, 50, and 70°C as well as for pH stability at pHs 3, 5, 7 and 9. As shown in Figures 4 and 5, all three DEET isomers were stable with no significant degradation over 5 days. These results imply the applicability of the DEET isomers as mosquito repellents.

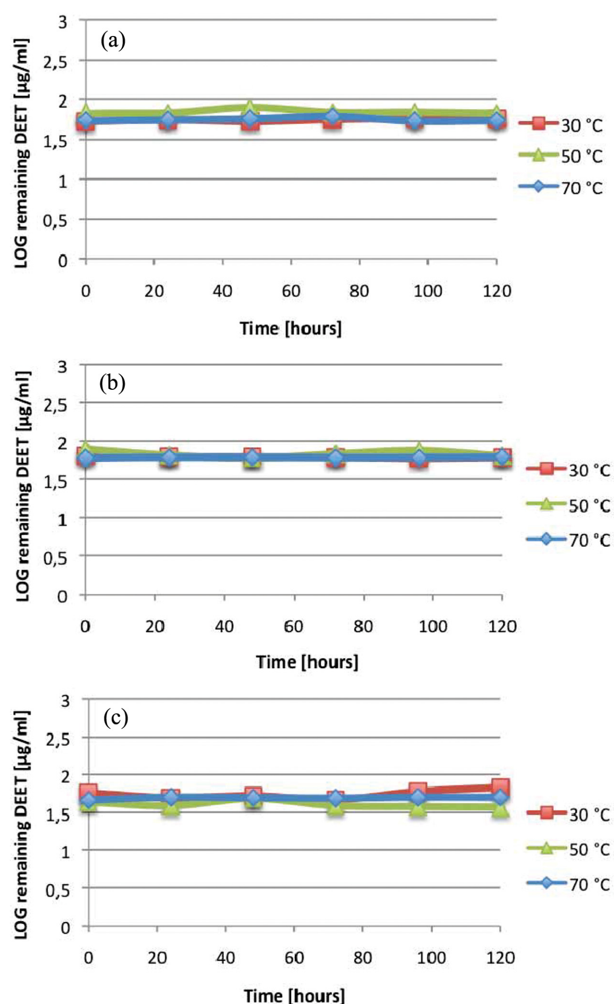

Figure 4. Temperature stability of (a) *ortho* DEET , (b) *para* DEET and (c) *meta* DEET at 30, 50, and 70°C.

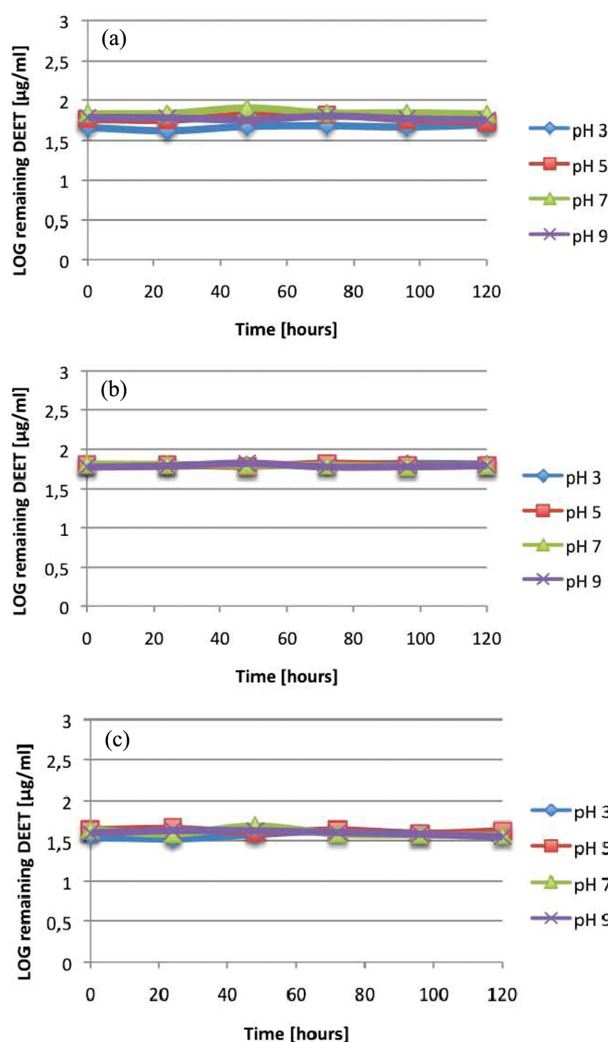

Figure 5. pH stability of (a) *ortho* DEET, (b) *para* DEET and (c) *meta* DEET at pHs 3, 5, 7 and 9.

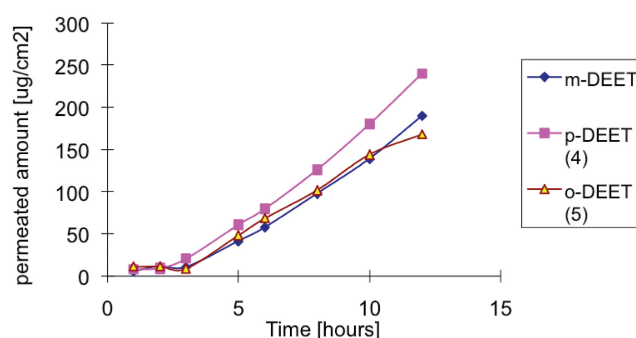

Figure 6. Cumulative amount of DEET analogues permeated per hour.

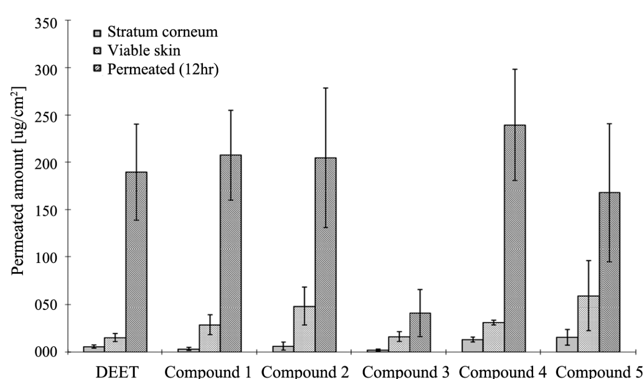

Figure 7. Skin deposition and permeation of DEET analogues. Comparison of deposited amount at the stratum corneum layer and viable skin layer with skin permeated amount of DEET and its analogues: Compound (1) 1-(3-methylbenzoyl)-pyrrolidine, (2) 1-(3-methylbenzoyl)-piperidine, (3) 1-(3-methyl-benzoyl)-piperazine, (4) *para* DEET and (5) *ortho* DEET.

Skin permeation and deposition study

Figure 6 shows the rat skin permeation profiles of DEET analogues in aqueous solution, using Keshary-Chien permeation cells at 37°C. After a time lag, the permeation of DEET through the skin followed a zero-order kinetics up to 12 hours. Although statistical significance was not shown at $p<0.05$, the *ortho*-DEET permeated the least as shown in the permeation rate of 16.54 μg/cm^2/hr in comparison to 22.99 and 28.39 μg/cm^2/hr of the *meta* and *para* analogues respectively.

Skin deposition and permeation profiles of the DEET analogues are shown in Figure 7. Compared to *meta*-DEET, Compound (1), 1-(3-methylbenzoyl)-pyrrolidine and Compound (2) 1-(3-methylbenzoyl)-piperidine exhibited similar permeation profiles as *meta*-DEET while Compound (3) alias 1-(3-methylbenzoyl)-piperazine permeated the least due to its high hydrophilic character (Jahn et. al., 2010). Compounds (4) and (5) which are *para*-DEET and *ortho*-DEET respectively, differ in their permeation as well as deposition profile although statistical significance was not shown at the $p<0.05$ level. The *ortho* analogue permeated less while the *para* analogue resembled the *meta* analogue better. According to a previous study, the complete protection time(CPT) in terms of mosquito repellency of the *ortho* and *para* analogues were longer than the *meta* analogue, indicating that the two were more effective than the commercially used *meta* analogue (Jahn et. al., 2010). The fact that the *ortho* DEET permeated the least indicates that the *ortho* analogue could exhibit less toxicity while maintaining the efficacy of the *meta* DEET. Moreover, the fact that the *ortho* analogue exhibited stability similar to the other two analogues makes it a feasible candidate for commercial use.

Conclusion

The three analogues, *ortho*, *para* and *meta*-DEET were stable at temperatures ranging from 30° to 70°C as well as in the

pH ranges of 3 to 9. The fact that *ortho*-DEET exhibited similar repellency to the commercially used *meta* analogue while permeating less through skin and existing in a solid state at room temperature, makes it an attractive candidate for a safer and more effective mosquito repellent.

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