AUTOMATED INSTRUMENTATION TO MEASURE EVAPORATION RATES OF REPELLENTS

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ABSTRACT. An evaporation apparatus was developed for *in vitro* evaluation of repellent evaporation from long-lasting mosquito repellent formulations. EVAP was used to evaluate mixtures of deet with vanillin and vanillin derivatives. Those formulations, which exhibited a lower evaporation rate of deet in EVAP studies, were found to afford a longer duration of protection against *Aedes aegypti* (L.) mosquitoes.

INTRODUCTION

The duration of protection afforded by a topical mosquito repellent is affected by several factors among which are the inherent repellency of the repellent molecule to mosquitoes, penetration and evaporation from the skin (Smith et al. 1963, Feldman and Maibach 1970, Spencer et al. 1974, and Gabel et al. 1976). For a given repellent like N.N-diethyl-m-toluamide, deet, (Gilbert et al. 1957) the inherent repellency will remain constant for a specific species of mosquitoes under a given set of physical conditions; however, many repellent formulations have been developed which prolong the protection period of deet. The increased persistence has been attributed to synergistic effects of additives (Khan et al. 1975) and controlled release from polymer formulations (Kurtz et al. 1973).

The current paper is a description of an automated evaporation apparatus (EVAP) which monitors the evaporation of deet from formulations, and an illustration of how EVAP is used to evaluate deet formulations with vanillin derivatives. Comparison of the evaluation using EVAP to actual *in vivo* trials of the formulations against mosquitoes will provide an explanation of the mechanism by which vanillin increases the protection period of deet.

EVAPORATION APPARATUS (EVAP). The rate of deet evaporation was determined by spreading 1.0 mg/cm^2 of ^{14}C-labeled deet evenly over an aluminum planchet, which was then placed between the two halves of the evaporation apparatus (EVAP) chamber. Collections were taken hourly and counted in a scintillation counter. The same procedure was followed using vanillin and vanillin derivatives as additives in a fixed ratio by weight with deet. A schematic diagram of the total EVAP system is shown in Figure 1. It consists of the following sub-systems: (1) an evaporation chamber, (2) a water bath for controlling the temperature of the evaporation chamber and the air flowing through it, (3) a primary bubble trap, (4) a toluene storage and pressurizing system to supply the bubble trap, and (5) a set of controls to govern the operation of the whole system.

A scored aluminum planchet with a surface area of 0.41 cm^2 is used as an evaporation surface. The planchet (H), which is attached to a Teflon® disc, is placed between two double walled glass halves of the chamber. The combined unit is inserted into an outer frame and a threaded cap is screwed up from the bottom to hold the whole assembly together. Water from the controlled temperature bath is circulated through spaces between the interior and exterior walls of two glass portions of the chamber to maintain a temperature of 32.2° C immediately around the evaporation surface. Air, which has also been preheated to 32.2° C, is introduced through a vertical tube directly above the planchet and flows directly down onto the

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[1] Reprint requests should be sent to the first author. The opinions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or Department of Defense.

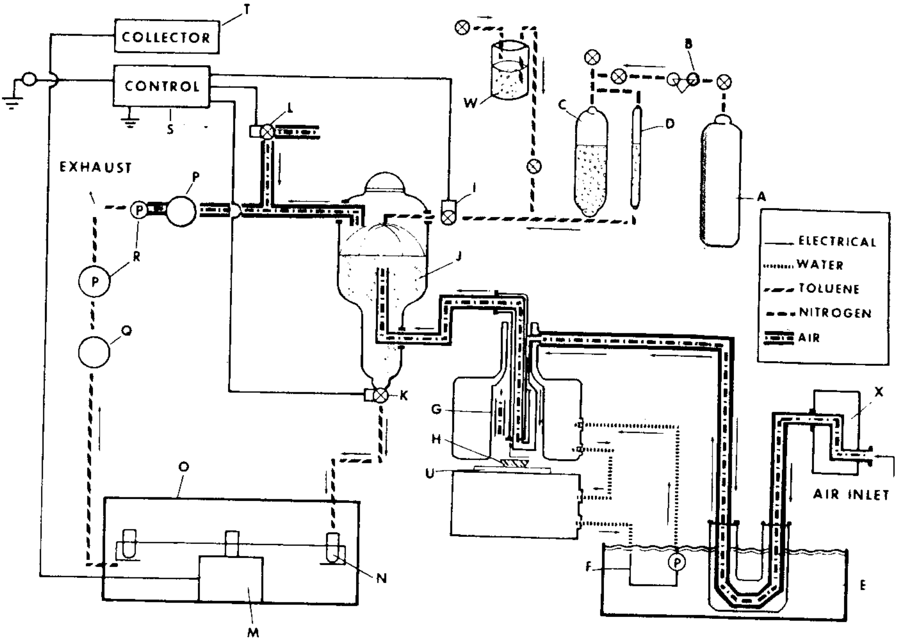

Fig. 1. Schematic diagram of repellent evaporation apparatus.

A	Nitrogen tank
B	Pressure regulator
C	Pressurized toluene reservoir
D	Toluene level gauge
E	Water bath
F	Pump, heater and circular unit
G	Evaporation chamber
H	Planchet
I	Toluene solenoid valve
J	Separatory funnel trap for repellent
K	Repellent trap release solenoid valve
L	Vent solenoid valve
M	Fraction collector
N	Sample in liquid scintillation vial
O	Enclosure for fraction collector
P	Secondary trap
Q	Trap for ventillation of fraction collector enclosure
R	Air pump
S	Main control box
T	Fraction collector control box
U	Teflon ® disc
W	Unpressurized toluene reservoir
X	Desiccator

sample, then radially out from its center, and back up along the outside of the same tube.

Air leaving the evaporation chamber is bubbled through a separatory funnel (J) containing toluene to trap evaporated repellent. Air leaving the first trap is passed through a second trap (P) to be certain of removing all the radio-labelled compounds before air is discharged to the atmosphere. This second trap can also be used to check efficiency of the first trap (in normal use, the first trap removes all the measurable radioactivity). At the bottom of the primary trap, an electrical solenoid valve (K) discharges toluene at fixed intervals. Each discharged toluene sample is channeled to a liquid scintillation vial (N) located in a fraction collector (M). Since the vial remains uncapped, the entire fraction collector is enclosed in a box (O) to prevent the evaporation of radioactive compounds into the atmosphere. A small flow is maintained through the enclosure with discharged air bubbled through a trap containing toluene (Q) that is identical to the other secondary trap (P). Both flow of air through the evaporation chamber (G) and flow through the fraction collector enclosure (O) are pumped by a dual head Masterflex® Pump (R). Induced flow was used in both cases for safety consideration; if a leak should develop, air will be drawn into the system rather than expelled from it.

Once the toluene sample has been discharged from the separatory funnel trap (J), the trap is refilled from a toluene reservoir and pressure system. A nonpressurized 5-gallon reservoir (W) is used periodically to fill a smaller (3000 ml) container (C), pressurized at 5 psig by a bottle of compressed nitrogen (A) with a regulator (B). Discharge of toluene from the pressurized container is controlled by an electrical solenoid valve (I).

The total system contains three solenoid valves: a toluene control valve, a trap discharge valve, and a venting valve (L) to stop and start air flow. It is important that flow be stopped whenever the system is not closed to the atmosphere such as when the trap is being discharged or filled. The venting valve (L) is located so that it stops flow by breaking suction and thus acts as a vent to the primary trap during the filling and discharging processes.

Coordination of the three solenoid valves and the fraction collector is accomplished with an electrical control system (S), composed of a timer built into the fraction collector (T), five other variable short period timers, and a relay to control time sequences of a repeating cycle. The fraction collector timer controls length of collection period and initiates the cycle by rotating the fraction collector to the next vial. A signal is taken from the collector during rotation which causes a delay to open a trap valve and start a cascade of five variable timers 2½ seconds later. Variable timers then control (1) a trap discharge period, (2) a trap rinse period, (3) a line purge period, (4) a rinse discharge period, and (5) a trap refill period. The duration of each period can be set on a dial for each respective timer. Toluene level in the separatory funnel trap is controlled by adjusting the refill timer. By using a cascade technique, i.e., one timer turns on the next timer, length of one period can be adjusted without affecting length of the other periods. Each variable timer has the capability of closing or opening three switches, one of which is operated slightly before the others, for example, making it possible (1) to stop air flow with vent (L) just before (2) discharging toluene trap (J) without using an additional timer. The control box also contains over-ride switches to allow an operator to control the system manually.

REPELLENT TESTS. Repellents and additive chemicals used in these studies were N,N-diethyl-M-toluamide (deet), 95% meta-isomer, practical grade; 3-methoxy-4-hydroxybenzaldehyde (vanillin), practical grade; 3, 4-dimethoxy-benzaldehyde (DMB), practical grade; p-hydroxy-benzaldehyde (PHB), practical grade; and benzoylphenylcarbinol (benzoin), practical grade.

Repellent formulations in ethanol solution were applied to 7x10 cm sites on the ventral forearms of test subjects such that the applied dose of deet would be 0.16 mg/cm^2. Subjects were tested in groups of 4 with 4 repellent-treated sites (two on each forearm) per individual (Brodel 1974). Application of formulations was rotated among the four sites so that each repellent appeared on a different site among the four subjects. Data were analyzed by paired comparison with the deet control using Tukey's w-procedure (Ostle 1963). Subsequent to application, the subject inserted his arm into a plastic sleeve with holes corresponding to the repellent-treated sites and introduced his forearm into a cage containing 250 active female *Ae. aegypti.* for a 3-minute exposure. The test was repeated at hourly intervals until two bites were received on a treated site. Then that site was covered; the protection time was recorded; and testing continued until all sites had failed.

Healthy, active-duty military personnel were selected at random from a volunteer population of 30 males, averaging 22 years in age (range 20–28). All volunteers gave written, informed consent prior to participation in the tests.

Mosquitoes were maintained under constant light at 27° C and 80% relative humidity with a 5% sucrose solution in cotton feeders continuously available. Female mosquitoes 7–10 days old were removed from the stock cages with a collecting tube and placed in test cages one day before repellent tests.

RESULTS

A typical curve (Fig. 2) for the evaporation of deet is shown using EVAP. Characteristically, this curve has a steady state plateau representing a constant evaporation rate of repellent, yielding to a rapidly descending slope. Reproducibility of this plateau is seen in the results shown in Table 1. From the height of this plateau and the time at which descent occurs, one can estimate efficacy of a deet formulation. Addition of vanillin to deet in a 1:1 ratio by weight reduces overall evaporation rate, permitting an extension of protection time

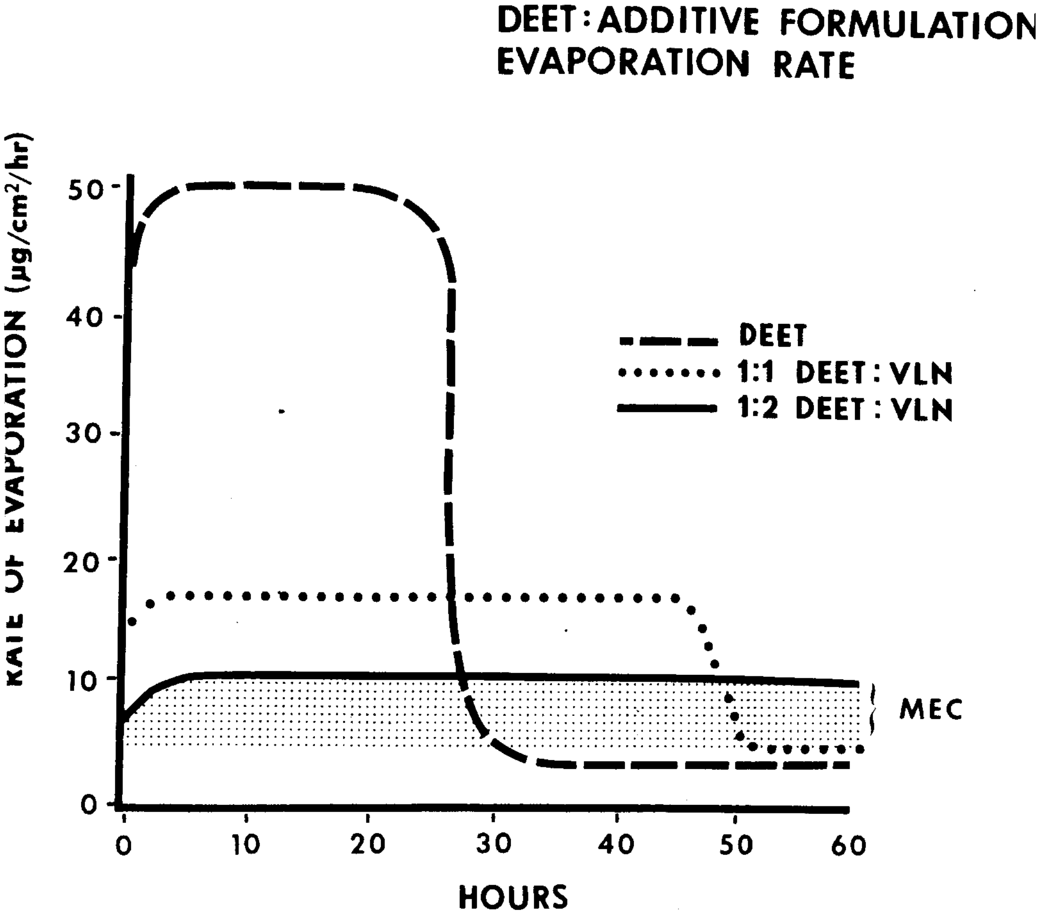

Fig. 2. Plot of evaporation rate of deet from a scratched planchet at an air flow of 30 cc/minute in the EVAP apparatus. Deet (— —), deet:vanillin (1:1) (. . . .), deet:vanillin (1:2) (——). MEC is the minimum effective concentration of repellent in the air above the skin necessary to prevent the target arthropod from biting the host.

Table 1. EVAP results for a deet standard solution.

Date	Plateau rate ($\mu g/cm^2/hr$)	Duration of plateau (hr)
11 Jul 73	50	25
23 Jul 73	50	25
26 Jul 73	55	21
8 Aug 73	52	23

afforded by the formulation (Fig. 2). The effect of different ratios of vanillin derivatives on the evaporation rate of deet is seen in Table 2. DMB, a methoxy derivative of vanillin, yields results similar to vanillin on the EVAP, while benzoin is somewhat less effective in controlling deet evaporation.

Table 2. *In vitro* evaporation of deet with additives.

Repellent:	Additive	Ratio	Number of Runs	Steady State Evaporation Rate $\bar{x} \pm s_d$ ($\mu g/cm^2/h$)
deet	....	....	4	53.0 ± 2.4
deet:	vanillin	1:1	2	15.5 ± 0.5
		1:2	3	9.7 ± 1.5
deet:	DMB	1:1	2	16.8 ± 6.0
		2:1	1	25.0
deet:	benzoin	1:1	1	<1
		2:1	1	1.5
		6:1	1	55.0

The results of protection tests against *Ae. aegypti* indicate that formulations of deet with vanillin derivatives tend to prolong the effective duration (Table 3). Vanillin applied at 0.32 mg/cm^2 exhibited some repellency on 2 of 8 subjects, showing no repellency on the other 6 subjects. Benzoin formulations with deet were statistically no different from deet alone.

DISCUSSION

With vanillin derivatives as additives, the evaporation rate of a volatile repellent like deet (Gabel 1976) can be controlled as shown in EVAP studies (Table 2). Moreover, lowering the evaporation rate by using deet:vanillin and deet:PHB mixtures apparently increases the duration of protection (Tables 2 and 3). Although vanillin itself acted as a repellent on 2 of 8 individuals in these tests, in later tests vanillin applied at 0.32 mg/cm^2 protected only 1 of 32 subjects and then only for 2 hours. The effect of vanillin on deet: vanillin mixtures is greater than simple addition of deet and vanillin protection times (Table 3 and Khan 1975). One might refer to this phenomenon as a synergistic effect of vanillin on deet repellency; on the other hand, the results of

Table 3. Protection time against mosquitoes afforded by deet additive formulations.[a]

Repellent:	Additive	Ratio	Number of Subjects	Protection Time $\bar{x} \pm s_d$ (h)
deet	....	....	16	3.56 ± 0.93
....	vanillin	....	8	2.00 ± 3.21
deet:	vanillin	1:1	8	6.50 ± 2.93[b]
deet:	vanillin	1:2	12	8.92 ± 3.15[b]
deet:	PHB	1:2	4	12.0 ± 4.36[b]
deet:	benzoin	5:1	4	5.00 ± 0.58
deet:	benzoin	7:1	4	4.75 ± 1.50

[a] Applied deet concentration was 0.16 mg/cm^2; vanillin was applied at 0.32 mg/cm^2 when tested alone.

[b] Significantly greater than deet ($p < 0.05$).

the evaporation studies seem to imply that the additive simply lowers the evaporation rate of deet without increasing the inherent potency of the repellent.

In developing new formulations one can use EVAP to determine the best formulation of a *known* repellent to use in testing on man or animal, thereby reducing the exposure of human subjects to a whole series of different compounds. An example of this application is seen in the deet:benzoin formulations (Table 2). The minimum effective concentration (MEC) of repellent vapor released from the formulation which has been found to be necessary for repelling mosquitoes has been determined empirically to be 5–7 mg/cm^2/h on EVAP. With the benzoin formulations, deet:benzoin ratios of 1:1 or 2:1 provided steady state evaporation rates less than 2 μg/cm^2/h on EVAP. When the ratio was as high as 6:1 deet: benzoin, the steady state evaporation rate was similar to that for deet. Subsequent testing of deet:benzoin formulations *in vivo* against mosquitoes showed that, indeed, the protection time afforded by deet: benzoin formulations with 5:1 or 7:1 ratios was approximately the same as by deet alone. In other words, prior to testing *in vivo,* it could be predicted from the EVAP results that benzoin would not offer an extended protection period when used in combination with deet.

The limit in lowering the evaporation rate of deet as observed in the benzoin case applies to specific laboratory testing methods. In Figure 2 the MEC level is described by a region between 5 and 10 μg/cm^2/h. If different conditions such as more avid mosquitoes, different species, or significantly heavier mosquito populations were used in the *in vivo* tests, the evaporation rate of deet from the 1:2 deet: vanillin formulation might have been too low to repel the mosquitoes and, under those test conditions, might have afforded no protection from mosquitoes. Therefore, the steady state level from EVAP which defines an effective formulation must be adjusted to the level of mosquito exposure expected under actual use of the formulation.

The EVAP apparatus has proved to be an efficient method for screening formulations of known repellents with low manpower requirements. EVAP can be used to monitor evaporation of repellents from formulations for 45 hours unattended, then with no interruption to the experiment, be prepared for another 45 hours of operation. Since the preparation time is less than one hour, the operator is free to perform other functions during the monitoring period. Evaporation data resulting from EVAP analysis can be used to select the best combination of repellent and additive for further testing on animals or man.

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