A STATISTICAL EVALUATION AND ANALYSIS OF MOSQUITO REPELLENT COMBINATION

by

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SUMMARY

The present product development project was aimed at studying the synergism and/or antagonism amongst various known insect repellent actives with the view to formulating a multi-active repellent product with improved properties when compared to current single-active commercial products. Advanced statistical methods were used to identify synergism between individual active substances and to define a formulation as close as possible to the "ideal" formulation. Several mosquito repellent samples were prepared and sent to the South African Bureau of Standards (SABS) in Pretoria to test for their efficiency in repelling mosquitoes.

From the results of the repellency tests of the various active combinations, three actives were identified that showed promising signs of synergism. These actives were then studied in further detail to determine their optimum combination. In addition, it was shown that when using a natural flavourant as promoter and incorporating a slow-release agent into formulations for aerosols and lotions, a product is obtained that gives comparable levels of efficiency to current commercial products, but at much reduced levels of active loading.

Accelerated stability tests performed on the final combination of the three actives used in the final formulation showed no adverse reactions over a three-week study. These tests shall be repeated once the final application form (lotion, aerosol, etc) and product packing have been decided.

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PART A

CHAPTER 1

INTRODUCTION

Each summer, outdoor loving people flock to their favourite destinations fully laden with the latest insect repellent products in order to prevent mosquitoes from biting. Besides the irritation of being bitten by mosquitoes, the threat of diseases such as Malaria, Yellow Fever and West Nile Virus is a constant concern to travellers worldwide. In addition to their potential health impact, insects such as mosquitoes can have a very significant economic impact on sectors such as the tourism industry.

Commercially available insect repellents are virtually all based on single active components. The latter can be divided into two categories: synthetic chemicals and natural plant-derived essential oils [1]. Repellents containing synthetic chemicals often have side effects, which make their use at higher concentrations undesirable. Limiting the upper concentration levels of such active components naturally affects the efficacy of the final formulated product. In the case of repellents based on plant-derived essential oils like oil of citronella (a mixture of geraniol, citronellal, borneol, methylheptenone and acetic and valerianic acids), they are not sufficiently effective[1], and high concentrations are required which may make their odour not well tolerated by users [2].

In view of the above considerations, there is a continual quest for the "perfect" topical mosquito repellent that is safe to use, effective and long lasting. During the 1940's, in their search to provide an insect repellent composition that was able to repel a wide range of biting insects, Bernard Travis and Howard Jones [3]

[1] Effective: producing the intended result

found that an insect repellent composition containing more than one known active (dimethyl phthalate, 2-ethyl-1,3-hexanediol and n-butyl mesityl oxide oxalate) provides better repellency[2] compared to compositions having a single active insect repellent. This observation is due to the additive effect of the actives, known as synergism. Synergism is the interaction of two or more agents so that their combined effect is greater than the sum of their individual effects. It originated from the Greek word *sunergos* meaning "working together" [4]. Despite this early evidence of synergism between insect repellent actives, it is rather surprising that very little has been described in the open and patent literatures that explores the possible synergism between such actives [3].

The primary objective of this study was to investigate the existence, or otherwise, of synergism between a number of selected repellent actives. The motivation for undertaking such a study was that if a synergistic effect between multiple actives exists, it may be possible to not only reduce the total amount of active in the final product, but also the risks normally associated with single active mosquito repellents. A further advantage is the reduction in the costs of raw materials used in such formulations.

This investigation will not include a study of the mechanism of synergism between the actives used in the mosquito repellent combination.

1.1 BACKGROUND

The outbreak of World War II and the need to conduct operations in areas where tropical diseases were common, led to the start of extensive investigations aimed at finding effective mosquito repellent actives. During these investigations, more than 4000 different compounds were screened for their efficacy in repelling a

[2] Repellency refers to a significant decrease in the number of insects probing or biting human skin where insect repellent has been applied compared with skin where repellent has not been applied.

variety of insects, including mosquitoes [5]. In the mid 1950's, researchers in the USA discovered that N,N-diethyl-*meta*-toluamide (or DEET) was highly effective for repelling biting insects. DEET remains the most widely used synthetic insect repellent today, being the active ingredient in about 200 commercial insect repellent products [6].

Malaria, often referred to as the "silent killer", is the world's most serious tropical disease and imposes very significant economic costs (associated with preventative measures, treatment, loss of work hours, loss of income, etc.) on countries like South Africa. The direct and indirect cost in Africa is estimated to exceed R20 billion a year [7]. Each year in Africa, mosquitoes inject malaria parasites into humans billions of times. As a result, some 300 to 500 million full–blown cases of malaria occur, and between 1 and 3 million people die. This is unlikely to be an accurate figure since most malaria deaths are not formally registered. It appears as though the malaria death toll rivals that of AIDS, which now kills about 3 million people annually [8]. Ninety percent of the deaths due to malaria occur in sub-Saharan Africa, and most are children under the age of five.

During the year 2000, over 62 000 cases of malaria were recorded in South Africa. The most seriously affected area was Kwazulu-Natal, which recorded the worst malaria epidemic since 1931. Upon investigating the sudden epidemic, the mosquitoes collected were identified as Anopholes funestus, a species prevalent in southern Mozambique and eradicated in South Africa during the 1950's [9]. The recent high rainfalls, the increase in labour migration, a reduction in the use of DDT and global warming are some of the factors said to be responsible for the sudden return of the Anopheles funestus mosquito [10]. Malaria is caused by the parasite of the genus plasmodium, which is carried by the female mosquito of the Anopheles species. Of the estimated 380 mosquito species in the genus Anopheles, only about 60 are able to transmit the malaria parasite to people.

Malaria transmission can be prevented or reduced through different control methods. The most direct and obvious precaution is to avoid getting bitten. Outdoor activities such as sports events, fishing and camping can become extremely unpleasant in areas where mosquitoes prevail. Consequently, mosquito repellents have occupied an important segment of the consumer health care market in many parts of the world.

1.2 PREVENTATIVE MEASURES

Attempts to control or even eradicate nuisance insects such as mosquitoes worldwide have been largely unsuccessful, and such an approach is environmentally questionable. Alternative methods of protection such as vaccination and preventative medication, while highly effective in certain cases, are very costly and only specific to any one type of disease. Insect repellents, on the other hand, have the advantage of providing protection against a variety of different insects, and hence also the transfer of disease. As a result, insect repellent products have developed into a very important sector of the consumer health market – not only because they are effective, but also because they are affordable. The main disadvantages of such products, however, are that they often only provide protection for limited time periods, thus requiring repeated application, and that the active ingredients, despite the use of fragrance materials, are often irritating to individuals.

There are many different methods of preventing mosquitoes from biting. However, the focus of this work is on repellents, *i.e.*, those substances applied to the skin that effectively prevent mosquitoes from biting. Protection from biting mosquitoes is best achieved by avoiding infested habitats, wearing protective clothing and using mosquito repellent products. However, in many circumstances, the latter protection method may be the only feasible way to protect against mosquito bites. While scientists may not fully understand how biting insects find their host, mosquitoes, the best studied of the biting insects, are known to use visual, thermal and olfactory stimuli to locate a blood meal [11].

It is believed that mosquito repellent actives, such as DEET, prevent mosquitoes from landing and biting their host because the active disturbs the function of special receptors found on their antennae that sense chemicals excreted from the skin [12].

The application of natural, strong-smelling substances to the human skin to repel mosquitoes and other biting insects dates back to ancient Egyptian times. In the Roman history, we find references to the use of substances such as camphor, cypress, galbanum, lupin and cinnamon for the same purpose. Since then, many compounds have been tried and tested for repellence efficiency including garlic, olive oil, pennyroyal oil, raw tomato juice and many more. While some natural extracts are capable of providing some degree of protection against certain insect bites, they normally have a number of disadvantages:

- They provide protection for very short periods of time;
- They need to be used in very high concentrations in order to be effective;
- They are not well tolerated by users (most have an unpleasant smell)[13].

As a result of these limitations, there has been an extensive effort from scientists and entrepreneurs to find the so-called “magic repellent compound”, *i.e.*, a single compound that would not suffer from the above-mentioned shortcomings. While some very effective compounds have indeed been discovered (and are used extensively in repellent products today), they also do not fully circumvent these shortcomings. In addition, such compounds have brought along there own unique problems.

In order to minimize inconsistencies in product performance testing of insect repellents, guidelines were set by the Environmental Protection Agency (EPA) in the United States [14]. Other requirements for an effective insect repellent against blood-feeding insects such as mosquitoes are:

- Complete freedom from toxicity and irritation when applied regularly to a person’s skin;

- Cosmetic acceptability including freedom from unpleasant odour or touch, and harmless to clothing;
- Protection against all biting mosquitoes;
- Cost effectivity and ready availability [15].

1.3 CURRENT TRENDS

In an effort to repel mosquitoes, consumers spend large amounts of money annually on sprays, lotions, liquids, candles and personal mosquito repellers[3]. All modern repellent products contain an active ingredient, one or more solvents and, in most cases, a fragrance material to mask the unpleasant smell of the active ingredient. After application to the skin or other surface such as clothing, the solvents in the repellent evaporate, leaving a protective layer that interferes with the normal "scent" provided by the body. The repellent will then only be effective for as long as it takes the active component in the repellent to evaporate or be removed by other mechanisms such as washing, sweating, etc. Most repellent actives are high-boiling liquid compounds with boiling points above 150^{o}C, implying a low susceptibility to facile evaporation.

The majority of effective insect repellent products on the consumer market contain DEET as the active ingredient. Despite its effectiveness, DEET has a number of disadvantages, namely:

- It has a high potential to irritate eyes and mucous membranes;
- It has a sticky, greasy feeling on the skin;
- It has a strong, long lasting odour [13].

These negative attributes lead to instinctive rejection of DEET-containing products by many consumers. Products containing DEET are not recommended for continuous use or for use on infants since DEET is suspected of causing, amongst other medical conditions, meningitis. DEET has a strong solvent and

[3] Repellers refer to items such as wristbands impregnated with insect repellents.

plasticiser effect on many plastic items and lacquered surfaces, and can cause severe damage to glasses, watches and other synthetic materials used in clothing and accessories. In light of this, there is a significant demand for an effective mosquito repellent active that poses no adverse effects, *i.e.*, a DEET-free product

In South Africa, almost all the synthetic mosquito repellent products contain DEET as the active ingredient, despite the possible health risks referred to above. Natural mosquito repellents tend to contain oil of citronella as the main repellent active. There are, however, a number of reports comparing data on the efficacy of such products with their synthetic alternatives. The results from such products tested under rigorous laboratory conditions have shown that claims of their effectiveness is severely over-rated. In most cases, such products:

- Will work for disappointingly short periods, usually less than 2 hours;
- Will only provide protection against some insects;
- Will not protect against very aggressive insects, in particular mosquitoes;
- Have to be used in such high concentrations that they may be even more irritating than their synthetic counterparts [16].

In this study, a number of currently available products (Tabard, Peaceful Sleep and Mylol) was obtained in order to determine the insect repellent active used in their product range and the quantity of active present (Table 1.1).

Table 1.1: Repellents in the South African marketplace

Brand	Product	Active ingredient	% Active
Tabard	Stick	DEET	35
	Lotion	DEET	19.5
	Candle	Citronella	5
	Towelletes	Citronella	15
	Aerosol	DEET	15
Peaceful Sleep	Stick	DEET	35
	Aerosol	DEET	15
Mylol	Lotion	DEET, DMP, DBP	9, 57, 34
	Aerosol	DEET	15
	Roll-on	Citronella	15

1.4 SUMMARY

There is little doubt that insect repellent products are and will remain an important aid in preventing insect bites. There is, however, clear opportunities for improving such products to the benefit of the user and the manufacturer. This project will try and address some of these issues, which include *inter alia*:

- Improving the efficacy of products;
- Reducing the loading of actives in final products;
- Increasing consumer satisfaction with product properties (odour, skin feel, etc);
- Reducing the health risks associated with such products.

As stated previously, this project will attempt to achieve some or all of these goals by evaluating the existence of synergism between various potential actives that may result in improved efficacy. We have also made a decision to

specifically exclude DEET as an active from these studies in view of the reported drawbacks associated with this compound.

CHAPTER 2

EXPERIMENTAL

2.1 MATERIALS

All reagents and solvents (Table 2.1) were obtained from commercial sources and were used as received.

Table 2.1: Organic and inorganic reagents for synthesis, formulation and analytical procedures

Reagents for	Chemical name	Source	Grade/ purity
SYNTHESIS	Citronellal	Sharon Bolel	racemic
	Sulphuric acid	Merck	98 %
	Sodium hydroxide	Merck	AR
	n-Heptane	Merck	AR
FORMULATION	Dimethyl phthalate	Saarchem	97 %
	Benzyl benzoate	Merck	98 %
	2-Butyl-2-ethyl-1,3-propanediol	Aldrich	99 %
ANALYSIS	Methanol	Merck	HPLC
	1,4-Dichlorobenzene	Aldrich	AR

2.2 PROCEDURE FOR THE SYNTHESIS OF *p*-MENTHANE-3,8-DIOL

Into a 3000 cm^3 round bottom flask was charged 636 g (16.3 mmol) of a 0.25 wt % sulphuric acid solution. This mixture was heated to 55^0C, and citronellal (500 g, 3.24 mol) was added dropwise over 1 hour whilst stirring the solution. The reaction mixture was maintained at 55^0C for 10 hours. Sodium hydroxide (8 g, 50 mmol, 25 wt %) was then added, followed by 1200 cm^3 of n-heptane, and the mixture stirred efficiently. The organic layer was removed and washed with 500 cm^3 of deionised water. The water layer was separated, and the organic layer refluxed under azeotropic conditions to remove residual water. The organic layer was then distilled at 80^0C under 1 mm Hg of pressure to give *p*-menthane –3,8-diol (541.82g, 3.14 mol, 97% pure based on GC-peak area); EI-MS (*m/e,* relative intensity) 157 (M^+-15.4), 154(M^+-18.5), 139 (11), 121 (9), 111 (7), 96 (53), 81 (100), 67 (18), 59 (62), 54 (23), 43 (34).

2.3 PREPARATIONS OF FORMULATIONS

Into a 100 cm^3 beaker was weighed the required amount for the selected percentage ratio of each active ingredient to be included (see Tables 3.1 and 3.3) for each individual mixture. This mixture was heated at 50^0C until a clear liquid was obtained, and the required amount of commercial aqueous cream was then added to give a total sample mass as required. This mixture was efficiently mixed by stirring for 15 minutes.

2.4 ANALYTICAL PROCEDURES

2.4.1 GLC-Mass Spectrometry

GLC-Mass spectrometry was performed on a Thermo Focus gas chromatograph coupled to a mass selector detector. The GLC was equipped with an RX-35 MS capillary column (30 m $\times$ 0.25 mm i.d.). Helium was used as the carrier gas with a flow rate of 40 $cm^3.min^{-1}$ at a column head pressure of 1.5 psi. Data from the

detector was analyzed by means of a personal computer with Xcaliber V3 software. Details of the column temperature program are shown in Table 2.2.

Table 2.2: GC-MS temperature program

Parameter	Setting
Column initial temperature	70^0C
Initial hold time Heating rate	5 min 10^0C.min^{-1}
Column final temperature	270^0C
Final hold time	5 min
Injector temperature	280^0C
Detector temperature	280^0C
Detector solvent delay	1.2 min

2.4.2 Capillary Gas Chromatography

GLC analysis was performed on a Thermo Finnigan Gas Chromatograph equipped with a flame ionisation detector and a Supelco Alphadex-120 capillary column (30 m $\times$ 0.25 mm i.d.). Data from the FID detector was analyzed by means of a personal computer equipped with DELTA Windows chromatography software. The carrier gas (N_2) flow rate was 5 cm^3.min^{-1} at a column head pressure of 10 psi. The split/splitless injector (model 1076) was operated at a split ratio of 1:70, and an injection volume of 1 µL was used. Samples were injected with the aid of an auto-injector (model A1 3000). Details of the column program used are shown in Table 2.3.

Table 2.3: GLC temperature program

Parameters	Setting
Column initial temperature	70^0C
Initial hold time Program rate	1 min 10^0C.min^{-1}
Column final temperature	270^0C
Final hold time	5 min
Injector temperature	250^0C
Detector temperature	280^0C

The internal standard method was used for all quantitative work with the internal standard being 1,4-dichlorobenzene. Response factors for the components of interest were determined by means of three calibration injections with known masses of standards and internal standard, prior to analysis.

This mode of operation was mainly used to determine the purity of citronellal and *p*-menthane -3,8-diol.

2.5 EFFICACY TESTING PROCEDURES

In accordance with the relevant method in Section 4 of SABS Method 807, yellow fever (Aedes aegypti) mosquitoes approximately 7-14 days old were deprived of a blood meal for >96 hours and then used as the test insects. The following equipment was also utilised for the repellency tests:

- Wooden test cages 300 mm high, 300 mm wide, 450 mm long and with sides covered with nylon mosquito netting, one side having a sleeve-inlet (Illustration 1);
- Plastic tubes 200 cm^3 with a diameter of approximately 60 mm and height 80 mm, and covered with nylon mosquito netting on both ends (Illustration 2).

The wooden test cages that were used were prepared 24 hours before commencement of the repellency test, and contained 100 mosquitoes; the 200 cm^3 plastic tube contained 30 mosquitoes and was prepared an hour before. As a food source, cotton wool was soaked in a 5 % sugar solution and placed inside the test cages and plastic tubes.

The selected volunteer's forearm was washed thoroughly with unscented soap and water to remove any traces of perfume. As a control to determine if the mosquitoes and arms used for the test were normal, one arm of each volunteer, chosen at random by the test officer, was placed inside a wooden cage containing 100 mosquitoes. Once 10 mosquitoes had landed, the time was recorded and the arm withdrawn from the cage. The norm used is that at least 10 landings should be recorded within a 30 second period. The hand of the volunteer was covered with a latex glove during the control test (Illustration 3).

Each volunteer's forearm was divided into three areas by drawing a line with a pen at the borders of each area. These areas were treated liberally with a numbered sample. The areas were treated by applying the sample first to the left arm area closest to the hand, the area next to this treated second, and so on, with the last treatment being closest to the right hand. After a fifteen-minute wait, either a plastic tube was placed on the treated area (Illustration 4) or the arm was exposed to the test cage (Illustration 5) for a five-minute period. The number of bites obtained during the five-minute period was recorded, and only those treated areas where five or less bites were recorded were re-exposed hourly for up to 5 hours.

Illustration 1: **Test cage containing 100 mosquitoes**

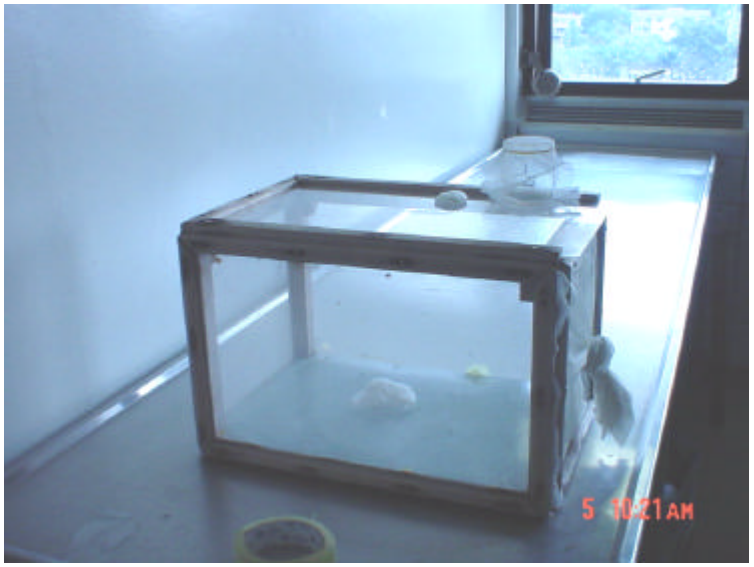


Illustration 2: **Containing the 30 test mosquitoes**

Illustration 3: **Control test**

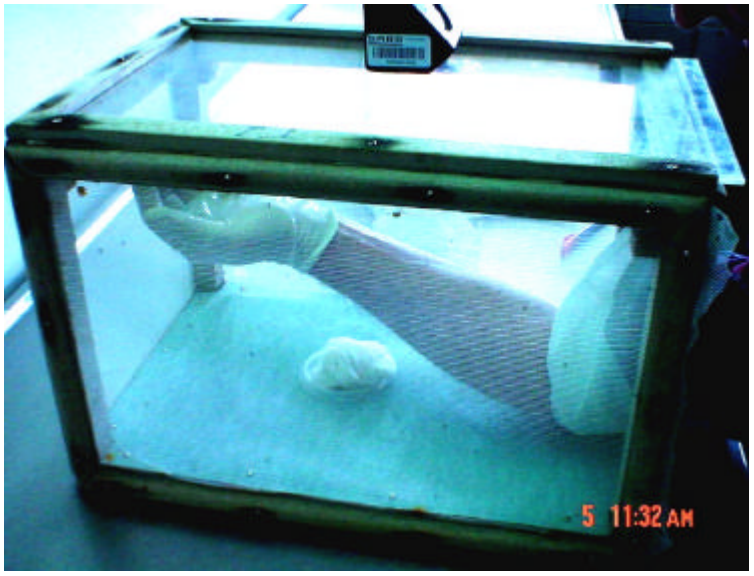


Illustration 4: **Treated area covered with the tube containing 30 mosquitoes**

Illustration 5: **Treated area placed inside cage containing 100 mosquitoes**

2.6 PROCEDURE FOR STABILITY TESTING

Three samples (Sample 1, Sample 2, and Sample 3) each containing 30 g of insect repellent actives, were made up in the following ratio:

p-Menthane-3,8-diol:	70 %
Benzyl benzoate:	15 %
2-Butyl-2-ethyl-1,3- propanediol:	15 %

The mosquito repellent actives were combined together into beakers and stirred for 10 minutes whilst purging with nitrogen gas. Three temperature settings were used in the stability tests, namely 26, 38 and 58^0C. For each of these three temperature settings, approximately 10 cm^3 of the mosquito repellent combination labelled Sample 1, Sample 2 and Sample 3 was placed into glass vials and sealed with plastic lids.

To ensure that the temperature remained constant, the temperature within the incubators was monitored weekly, using a standard 120^0C mercury thermometer.

Each week, an approximately 2 cm^3 sample was collected from each of the glass vials stored in the incubators, and these analysed on the GC-MS as 0.6 % methanol solutions.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 REPELLENCY TESTS

Four known and approved insect repellent actives were combined in different proportions in an attempt to determine if synergism exists between any two or more of the actives. Three of the four actives used are so-called synthetic compounds, not being available from natural sources. They were dimethyl phthlate, benzyl benzoate and 2-butyl-2-ethyl-1,3-propanediol. All of these actives have individually been reported to possess some degree of repellent action against various biting insects [15].

During the latter days of World War II, clothing was impregnated with dimethyl phthalate (DMP), or preparations containing dibutyl phthlate (DP) and benzyl benzoate as a means of repelling insects. Another product referred to as M-1960 was developed by the U. S. Army to meet their needs for protection against a broad spectrum of arthropod vectors[4] and diseases, and consisted of equal parts N-butylacetanilide, 2-butyl-2-ethyl-1,3-propanediol and benzyl benzoate [17].

The fourth active, *para*-menthane-3,8-diol (PMD or Quwenling), is a naturally occurring insect repellent used extensively in China since 1978. It occurs in small quantities in the leaves of Eucalyptus trees but can be synthesized from citronellal, one of the main constituents of citronella, an essential oil distilled from the leaves and stem of the Cymbopogon nardus plant [18].

Due to the high costs involved in repellency testing, only four samples containing varying amounts of the four selected insect repellent actives were evaluated to

[4] Arthropod vectors: disease-carrying insects for example mosquitoes.

determine if, in fact, they repelled mosquitoes. Once these results were obtained, more samples were formulated according to a specific mixture design, and sent for repellency testing. The results were statistically analysed and the best combination of actives identified.

Table 3.1 contains the details of the first batch of samples that was sent to the SABS Test House in Pretoria for preliminary repellency testing. Four different active combinations were prepared for these tests; each sample contained a total of 10 % (m/m) active (Table 3.1). Using the plastic tubes containing 30 female mosquitoes each, the treated areas were exposed for a 5-minute period only, to determine if a particular combination repelled mosquitoes. No bites were received for any of the samples during the 5-minute period and it was therefore possible to conclude that the four different combinations prepared did in fact repel mosquitoes (Table 3.2).

Table 3.1: Preliminary samples: Active loadings

No.	*p*-Menthane-3,8-diol	Dimethyl phthlate	Benzyl benzoate	2-Butyl-2-ethyl-1,3-propanediol	Aqueous cream
1	5 % (0.9945 g)	5 % (1.0169 g)	0	0	90 % (18.7272 g)
2	5 % (0.9964 g)	0	5 % (0.9512 g)	0	90 % (18.2614 g)
3	5 % (0.9520 g)	0	0	5 % (0.9560 g)	90 % (19.6458 g)
4	5 % (0.5139 g)	2.5 % (0.5102 g)	2.5 % (0.5102 g)	2.5 % (0.5614 g)	90 % (19.0090 g)

Table 3.2: Number of bites recorded during 5-minute exposure period

Sample No.	No. of bites
1	NONE
2	NONE
3	NONE
4	NONE

In view of the promising preliminary results obtained, it was decided to use statistical experimental design to establish unequivocally whether any synergism existed between any of the actives and also to obtain some indication as to the optimum ratios such actives should be combined in.

3.2 INTRODUCTION TO MIXTURE DESIGNS

Virtually all consumer products are mixtures of two or more ingredients. In all such cases, one or more particular properties of a specific consumer product are of particular importance to the user. For example, in the case of the headache tablet, properties such as the speed of action and the length of action may be important. The properties (effect) of mixtures/products are a direct result of:

- The type of ingredients; and
- The relative amounts (proportions) of the ingredients contained in the product.

Both the type and the relative amount of ingredients can profoundly influence the property or effect of a particular mixture. For example, a very effective herbicide (weed killer) may be practically useless if it cannot “wet” the surface of weed leaves. A surfactant may be used to aid the wetting process, thereby enhancing the effect of the herbicide chemical considerably. For existing products, the type of ingredients is normally fixed and the only way to enhance desirable properties,

or to mask undesirable properties, is to vary the relative proportions of the individual ingredients. Naturally, for new mixtures/products, the type of ingredient as well as the relative amounts of ingredients can be chosen to give the most desired effect.

The process of finding the best combination of proportions of ingredients is often a tedious one, carried out by persons with many years of practical experience in the particular field. When such experience is not available, the optimisation process often takes the form of trial and error, or “scatter-gun” procedures. These procedures are not only expensive in terms of time and materials, but result in considerable uncertainty in terms of whether the best possible combination has actually been achieved.

However, the experimental design procedures used in this study, which were first introduced in 1958 by Scheffé, are not only able to cover the entire range of possible combinations of components, but can also evaluate the best ratios in which the most promising components should be combined in [19].

3.3 DETAILS OF THE MIXTURE DESIGN USED

For the purposes of this investigation, a Simplex Centroid design in four factors was used. Five of the combinations were replicated in order to obtain an estimate of the experimental error in the efficacy test. The design (in coded format) is shown in Table 3.3.

Table 3.3: Simplex Centroid design used

Std	*p*-Menthane-3,8-diol	Dimethyl phthlate	Benzyl benzoate	2-Butyl-2-ethyl-1,3-propanediol
1	1	0	0	0
2	0	1	0	0
3	0	0	1	0
4	0	0	0	1
5	0.5	0.5	0	0
6	0.5	0	0.5	0
7	0.5	0	0	0.5
8	0	0.5	0.5	0
9	0	0.5	0	0.5
10	0	0	0.5	0.5
11	0.33	0.33	0.33	0
12	0.33	0.33	0	0.33
13	0.33	0	0.33	0.33
14	0	0.33	0.33	0.33
15	0.25	0.25	0.25	0.25
16	0.62	0.12	0.12	0.12
17	0.12	0.62	0.12	0.12
18	0.12	0.12	0.62	0.12
19	0.12	0.12	0.12	0.62
20	0	0	0	1
21	1	0	0	0
22	0	1	0	0
23	0	0	1	0
24	0.5	0	0	0.5

Details of the actual amounts of actives and commercial aqueous cream that were mixed to give the combinations specified by the above design are given in Table 3.4. Table 3.4 also gives a "score" for each formulation and the "run" refers to the actual sample number. The score figures will now be explained. Due to the large number of samples, twelve volunteers were used during the repellency testing. Each sample was replicated four times using a different individual in order to average (or allow for) the expected difference in response, resulting from the natural difference between the individuals used. Each individual's treated area was exposed to a test tube containing 30 mosquitoes, and once an accumulated amount of five bites had been received, the test was terminated for that sample. The result is that for some individuals the test was terminated before other individuals, which made interpretation of the results somewhat difficult. In the case of this design, it was therefore decided to evaluate the results as follows. Whenever a test was terminated for an individual, an arbitrary number of bites (15) were allocated to those times for which the sample was not tested. The total number of bites over a four-hour period for each sample was then added together and averaged over the four individuals. In so doing, the difference between individuals was "naturally" incorporated into the final evaluation, and the evaluation could be performed over a constant test period, namely four hours.

Table 3.4: Design composition details

Std	Run	Score	*p*-Menthane-3,8-diol	Dimethyl phthlate	Benzyl benzoate	2-Butyl-2-ethyl-1,3-propanediol	Aqueous cream
16	1	31	6.25 % (3.1109 g)	1.25 % (0.626 g)	1.25 % (0.6291	1.25 % (0.6276 g)	90 % (44.7321 g)
3	2	272	0	0	10 % (5.0096	0	90 % (44.6462 g)
8	3	138	0	5 % (2.4631	5 % (2.5349)	0	90 % (44.7443 g)
13	4	64	3.33 % (1.6602 g)	0	3.33 % (1.6617	3.33 % (1.6575 g)	90 % (44.752 g)
24	5	29	5 % (2.5109 g)	0	0	5 % (2.4905 g)	90 % (45.2769 g)
7	6	102	5 % (2.5057 g)	0	0	5 % (2.4905 g)	90 % (45.2769 g)
12	7	123	3.33 % (1.6521 g)	3.33 % (1.657 g)	0	3.33 % (1.6499 g)	90 % (46.3701 g)
2	8	174	0	10 % (5.0114	0	0	90 % (45.0589 g)
23	9	296	0	0	10 % (5.0114	0	90 % (45.0589 g)
10	10	89	0	0	5 % (2.4922	5 % (2.4693 g)	90 % (46.725 g)
9	11	126	0	5 % (2.5012	0	5 % (2.4878 g)	90 % (46.725 g)
14	12	184	0	3.33 % (1.6819	3.33 % (1.6779	3.33 % (1.6631 g)	90 % (43.9579 g)
15	13	99	2.5 % (1.2615 g)	2.5 % (1.2672	2.5 % (1.2631	2.5 % (1.2513 g)	90 % (44.5637 g)
11	14	131	3.33 % (1.6600 g)	3.33 % (1.6557	3.33 % (1.6573	0	90 % (45.2657 g)

17	15	141	1.25 % (0.6400 g)	6.25 % (3.1194	1.25 % (0.6296	1.25 % (0.6311 g)	90 % (45.4848 g)
5	16	70	5 % (2.5013 g)	5 % (2.4220	0	0	90 % (44.5782 g)
4	17	84	0	0	0	10 % (5.0080 g)	90 % (45.4720 g)
18	18	101	1.25 % (0.6334 g)	1.25 % (0.6265	6.25 % (3.1420	1.25 % (0.6217 g)	90 % (44.749 g)
22	19	151	0	10 % (5.0181	0	0	90 % (45.4232 g)
6	20	37	5 % (2.5595 g)	0	5 % (2.5066	0	90 % (44.7134 g)
19	21	68	1.25 % (0.6351 g)	1.25 % (0.6365	1.25 % (0.6292	6.25 % (3.122 g)	90 % (46.0702 g)
1	22	43	10 % (5.0108 g)	0	0	0	90 % (44.6734 g)
20	23	57	0	0	0	10 % (4.9636 g)	90 % (45.2340 g)
21	24	75	10 % (4.9967 g)	0	0	0	90 % (44.8796 g)

The results of the design was analysed by Multiple Least Squares methods using Excel software and the following quadratic model was constructed:

$$\text{Response} = 54.54 \times A + 159.80 \times B + 275.86 \times C + 66.53 \times D - 56.48 \times AB - 472.03 \times AC + 22.95 \times AD - 180.51 \times BC + 155.56 \times BD - 275.99 \times CD$$

In the above model, A = *p*-menthane-3,8-diol, B = dimethl phtalate, C = benzyl benzoate, and D = 2-butyl-2-ethyl-1,3-propanediol. The terms AB, AC, etc. represent the interaction (synergism) between actives. The numerical coefficients in the model give an indication of the magnitude of the influence of that particular active, or combination of actives. It must be noted that in the present model,

lower score values are desirable (less bites). Before using the results of the model developed from the results of the efficacy tests, it was necessary to confirm that the model was statistically valid. This was done by means of an Analysis of Variance (ANOVA). Table 3.5 summarises the results of this analysis.

Table 3.5: Anova for Mixture Quadratic Model

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	93868	9	10429.8	10.2334	< 0.0001
Residual	14268.6	14	1019.19		
Lack of Fit	10175.1	9	1130.57	1.38093	0.3778
Pure Error	4093.5	5	818.7		

The above analysis shows that the model explains more than 99% of the variation in the results obtained, and hence the model is statistically valid and can be used for interpretation of the results, as well as prediction of possible optimum formulations.

Careful analysis of the above Quadratic model shows that:

- *p*-Menthane-3,8-diol and 2-butyl-2-ethyl-1,3-propanediol are the most effective as single active formulations;
- Benzyl benzoate is the worst single repellent active;
- There is very strong synergism between *p*-menthane-3,8-diol and benzyl benzoate (negative value of -472), and also between benzyl benzoate and 2-butyl-2-ethyl-1,3-propanediol (negative value of -276);
- While there is some synergism between dimethyl phtalate and benzyl benzoate, this synergism appears to be negated by antagonism beween dimethyl phtalate and 2-butyl-2-ethyl-1,3-propanediol.

The above conclusions are clearly supported by contour diagrams constructed by plotting the results predicted by inserting arbitrary values for the four variables (between 0 and 10%) into the Quadratic model. In each of the diagrams shown, one active was set to 0% so that the figures reflect the response in the variaton of only three factors.

Figure 3.1: Contour/response surface diagram
(*p*-menthane-3,8-diol + dimethyl phtalate + benzyl benzoate)

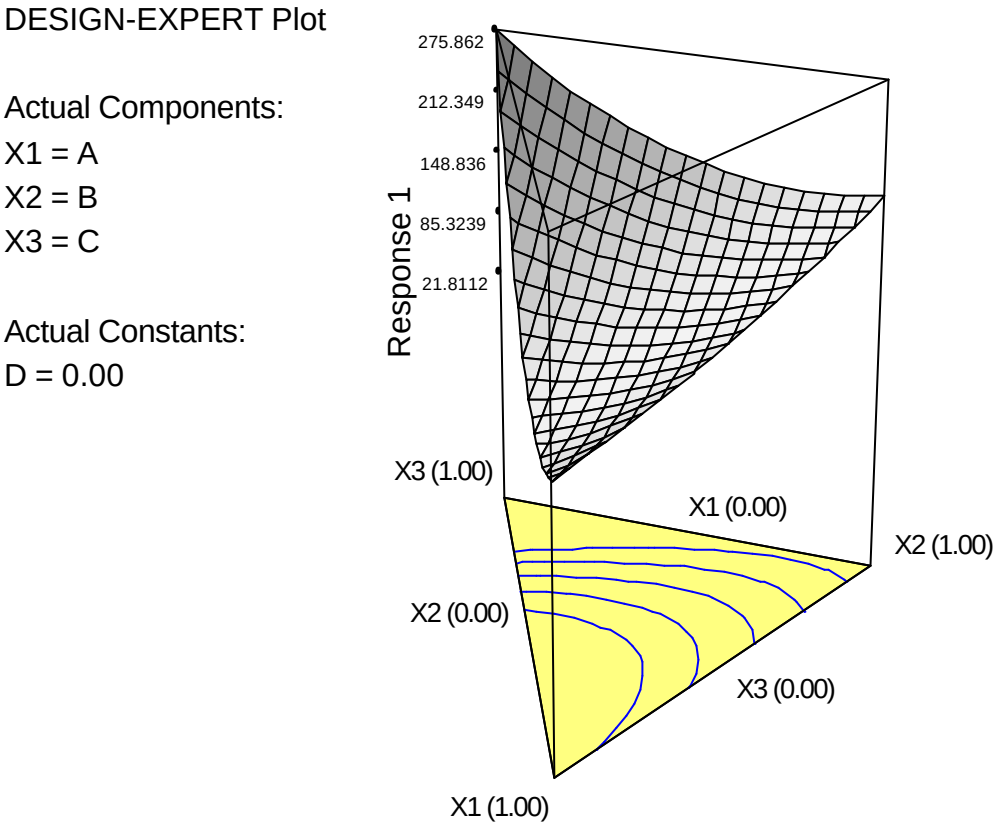


Figure 3.2: Contour/response surface diagram
(*p*-menthane-3,8-diol + dimethyl phtalate + 2-butyl-2-ethyl-1,3-propanediol)

DESIGN-EXPERT Plot

Actual Components:
X1 = A
X2 = B
X3 = D

Actual Constants:
C = 0.00

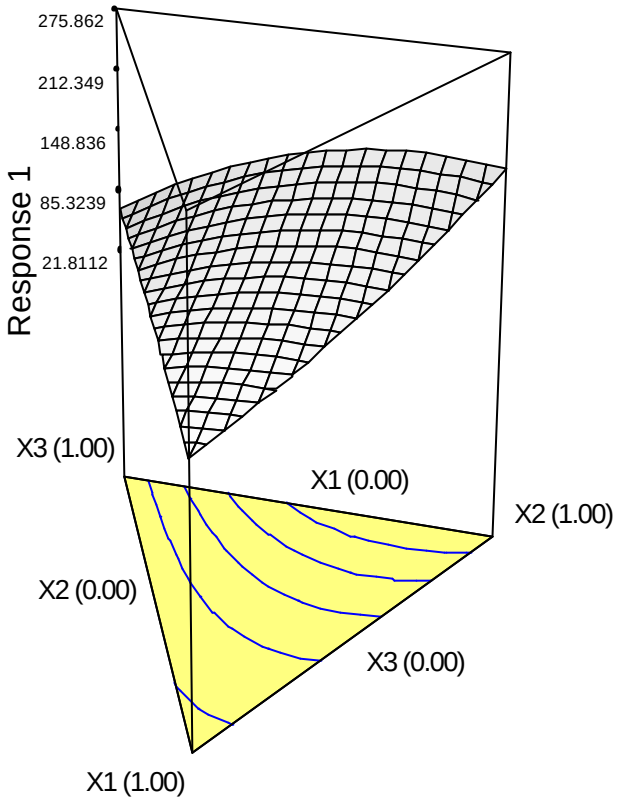


Figure 3.3: Contour/response surface diagram

(*p*-menthane-3,8-diol + benzyl benzoate + 2-butyl-2-ethyl-1,3-propanediol)

DESIGN-EXPERT Plot

Actual Components:
X1 = A
X2 = C
X3 = D

Actual Constants:
B = 0.00

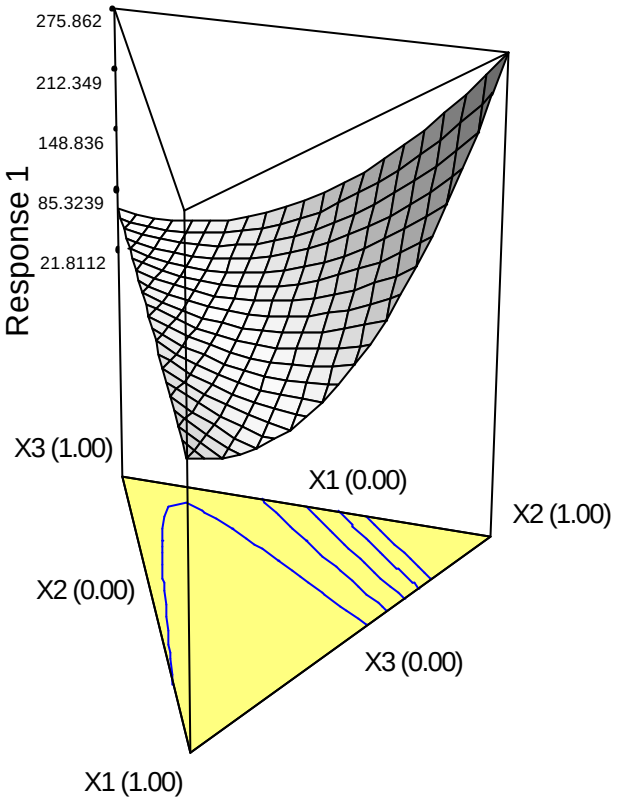
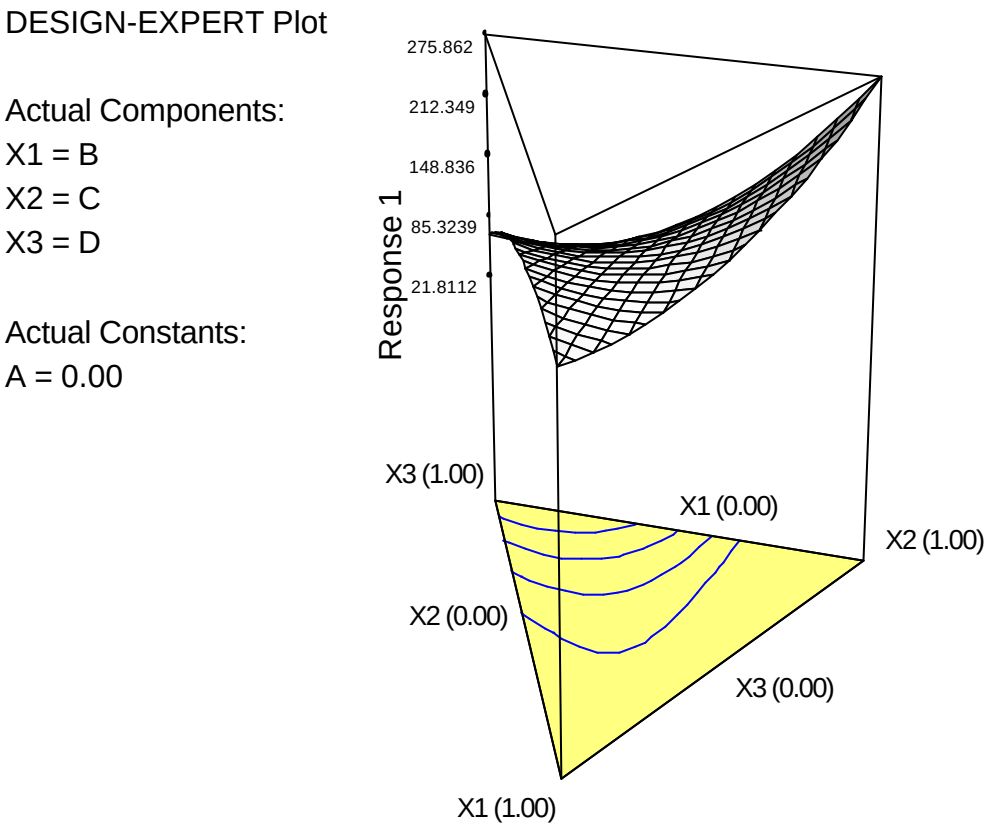


Figure 3.4: Contour/response surface diagram
(dimethyl phtalate + benzyl benzoate + 2-butyl-2-ethyl-1,3-propanediol)



From the above analyses, we can readily predict that dimethyl phtalate should be omitted from the optimum formulation (which is desirable in view of the reported toxicity dangers of dimethyl phtalate). Furthermore, *p*-menthane-3,8-diol should be the main constituent with minor amounts of benzyl benzoate and *2*-butyl-2-ethyl-1,3-propanediol. In order to fine-tune a possible final formulation, various values were inserted (in coded form) for *p*-menthane-3,8-diol, benzyl benzoate and 2-butyl-2-ethyl-1,3-propanediol into the Quadratic model. The predicted

response was calculated and it was noted that the response decreased as the active amounts were varied. Table 3.6 illustrates a number (from a much larger set of calculations) of these calculations (Note: The value of dimethyl phtalate was always set to zero to remove it from the formulation).

Table 3.6: Predicted values of the response as a function of varying amounts of *p*-menthane-3,8-diol (PMD), benzyl benzoate (BB), and 2-butyl-2-ethyl-1,3-propanediol (PD).

Actual amounts (%)			Coded values			Response
PMD	**BB**	**PD**	**PMD**	**BB**	**PD**	
10	0	0	1	0	0	54.54
9.5	0.25	0.25	0.95	0.025	0.025	49.53
9	0.5	0.5	0.9	0.05	0.05	45.31
8.5	0.75	0.75	0.85	0.075	0.075	41.86
8	1	1	0.8	0.1	0.1	39.19
7.5	1.25	1.25	0.75	0.125	0.125	37.29
7	1.5	1.5	0.7	0.15	0.15	36.18
6.5	1.75	1.75	0.65	0.175	0.175	35.84
6	2	2	0.6	0.2	0.2	36.28

The final combination of actives selected on the basis of these calculations were:

p-menthane-3,8-diol:	70 %
benzyl benzoate:	15 %
2-butyl-2-ethyl-1,3-propanediol:	15 %

To confirm that the above combination is an effective repellent for mosquitoes, several samples having the above ratio of actives were prepared and tested for efficacy. Unfortunately, the results of these tests were not conclusive as the

SABS Test House experienced problems with the test mosquitoes. During this time, talks were held with Durotek, a Port Elizabeth manufacturing company which specialises in biocide formulations. They expressed an interest to become involved in the project, particularly since they believed that the slow-release carrier medium they were using in their products could also be of benefit to products such as insect repellents. Durotek's formulation pharmacist subsequently prepared several new formulations using the final ratio combination of actives as defined from the results of the mixture design, the slow-release carrier, and also a small amount of flavourant as an enhancer.

In order to determine the best medium for the active combination, various samples were thus prepared using either an in-house aqueous cream or an alcohol-based solution. All of these samples contained the slow-release carrier but only some contained flavourant. Included in the sample batch were two samples containing 15 % DEET as active. The samples were tested for efficacy using test cages containing 100 mosquitoes, and once an accumulate number of 5 bites was recorded, the test was terminated. The results of the best replicates of these screening tests are summarized in Table 3.7.

Table 3.7: Results from efficacy testing

Sample number (contents)	Hours after treatment and total number of bites inflicted					
	1 hrs	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs
1 (DEET solution)	0	0	0	1	2	3
2 (DEET lotion)	0	0	0	0	5	
3 (solution)	2	2	0	3		
4 (lotion)	3	3				
5 (flavourant, solution)	0	0	0	0	4	1
6 (flavourant, lotion)	0	1	4			

The results in the table above clearly show that:

(i) The inclusion of the slow-release carrier markedly improves the efficacy of the repellent formulation;

(ii) The addition of the flavourant also enhances the efficacy of the repellent mixtures;

(iii) Alcohol-based combinations are more effective than aqueous cream-based mixtures;

(iv) Some of the mixtures tested are as effective as DEET, but contain less active (10% as opposed to 15% for DEET).

One problem observed with the new formulations, particularly with samples formulated in aqueous cream, was the oxidation of the added flavourant, which resulted in the mixtures turning black with time. It was clear that an additional addition, namely an antioxidant, would have to be added to the final mixture. In order to have some test samples prepared for the upcoming holiday season, it was decided to initially concentrate on the alcohol-based formulations as these could be readily packaged for distribution to volunteers for field-testing. In order

to counteract the observed oxidation of the flavourant, Vitamin E was added to these solutions and, provided samples were not overly exposed to air, oxidation was practically eliminated. A final sample containing the complete formulation, ready for packaging as an aerosol product (Table 3.8, Sample 2), was sent to the SABS Test House. The tests were done using, once again, the test cages containing 100 mosquitoes. However, the test period was reduced to a one-minute exposure time. The results of these tests were compared to a sample containing no repellent and a commercial insect repellent sample containing 19.5% DEET (Table 3.8). In view of the good results obtained, 100 aerosol samples were packed for distribution to volunteers in real-life field tests. The results of these voluntary tests are still outstanding.

Table 3.8: Comparative efficacy testing of aerosol sample

Sample number (contents)	**Hours after treatment and total number of bites inflicted**				
	1 hrs	2 hrs	3 hrs	4 hrs	5 hrs
1 (Lotion, vitamin E)	2	1	1	5	
2 (Solution, vitamin E))	0	0	4	3	7
3 (Solution)	0	1	1	5	
4 (DEET, lotion)	0	0	1	2	6
5 (No repellent)	45				

3.4 STABILITY TESTING

As part of the research, a controlled stability study was undertaken to determine how the individual insect repellent actives found in the final combination containing insect repellent actives *p*-menthane-3, 8-diol, benzyl benzoate and 2-butyl-2-ethyl-1,3-propanediol interact with each other over time at certain temperatures. It must be noted that these studies were conducted before Durotek's involvement; hence, these tests reflect the relative stability of the three

active compounds in the absence of a medium, slow-release carrier, flavourant and antioxidant.

A normal long-term stability study on any pharmaceutical formulation is usually done over a five-year period. However, due to time constraints, an accelerated time study over one month was conducted at specific storage conditions. An increase in temperature causes an increase in the rate of chemical reactions. For this reason, increased temperatures are normally used during such accelerated stability tests. For pharmaceutical substances, storage at room temperature is normally regarded as storing at 15^0C. However, under accelerated conditions, the required temperature must be at least 5^0C higher [20].

In, this specific study, the temperature was set at 26^0C, 38^0C and 58^0C. Since the nature of the final storage container for the mosquito repellent combination had not yet been determined at the time of this study, glass vials with plastic lids were used to store the samples for the duration of the stability test.

Each of the formulated samples were sampled and analysed on a weekly basis for four weeks. The analyses were performed by injecting known amounts of samples in the GC-MS and measuring the % peak area (of the total peak area as determined by the GC-MS) for each of the active components (Table 3.9). The peak areas for each individual active were then statistically analysed using Analysis of Variance (ANOVA) to determine if any significant decomposition or interaction had occurred.

Table 3.9: GC-MS results showing % peak area

Temperature	Sample no.	Time			
***p*-Menthane-3,8-diol**		T^0	T^1	T^2	T^3
26^OC	**S1**	67 %	62 %	47 %	53 %
	S2	64 %	72 %	50 %	47 %
	S3	63 %	67 %	52 %	48 %
38^OC	**S1**	67 %	63 %	61 %	50 %
	S2	64 %	57 %	68 %	50 %
	S3	63 %	64 %	62 %	52 %
58^OC	**S1**	67 %	57 %	60 %	50 %
	S2	64 %	58 %	57 %	52 %
	S3	63 %	60 %	53 %	51 %
Benzyl benzoate					
26^OC	**S1**	31 %	12 %	32 %	32 %
	S2	34 %	13 %	30 %	31 %
	S3	35 %	13 %	29 %	32 %
38^OC	**S1**	31 %	15 %	28 %	36 %
	S2	34 %	14 %	23 %	31 %
	S3	35 %	11 %	26 %	29 %
58^OC	**S1**	31 %	14 %	40 %	28 %
	S2	34 %	14 %	29 %	28 %
	S3	35 %	13 %	29 %	34 %

Table 3.9: continued

2-Butyl-2-ethyl-1,3-propanediol					
26^{O}C	**S1**	2 %	25 %	21 %	15 %
	S2	2 %	14 %	21 %	22 %
	S3	2 %	27 %	18 %	16 %
38^{O}C	**S1**	2 %	22 %	11 %	14 %
	S2	2 %	29 %	8 %	19 %
	S3	2 %	25 %	13 %	18 %
58^{O}C	**S1**	2 %	29 %	0 %	22 %
	S2	2 %	28 %	14 %	20 %
	S3	2 %	27 %	18 %	16 %

Table 3.10: ANOVA for *p*-menthane-3,8-diol

Source	Sum of Squares	DF	Mean Square	F Value	P-value	F crit
Sample	93868	2	23.36	2.79402	0.0811	3.4028
Time	14268.6	3	365.8	43.75083	6.95E-10	3.0088
Temp	10175.1	6	61.4	7.3387	0.0001	2.5081
Within	4093.5	24	8.4			

Table 3.11: ANOVA for benzyl benzoate

Source	Sum of Squares	DF	Mean Square	F Value	P-value	F crit
Sample	11.17	2	5.583	0.7256	0.4943	3.4028
Time	2287.67	3	762.556	99.105	1.17E-13	3.0088
Temp	73.5	6	12.25	1.592	0.1927	2.5081
Within	184.67	24	7.694			

Table 3.12: ANOVA for 2-butyl-2-ethyl-1, 3-propanediol

Source	Sum of Squares	DF	Mean Square	F Value	P-value	F crit
Sample	18.06	2	9.027	0.5652	0.5756	3.4028
Time	2532.78	3	844.259	52.8579	1.01E-10	3.0088
Temp	219.06	6	36.509	2.2858	0.06913	2.5082
Within		24	15.972			

Looking at the ANOVA table for *p*-menthane-3,8-diol (Table 3.10), we find that for samples S1, S2 and S3, the calculated value of *F*, referred to as $F^{\text{lack of fit}}$, is smaller than the critical value F^{crit} indicating that there is no significant difference between these samples. This is to be expected as these samples were all made up from the same batch. For the time variable, $F^{\text{lack of fit}} > F^{\text{crit}}$, confirming that some degradation occurred with time. Also, $F^{\text{lack of fit}} > F^{\text{crit}}$ for temperature, indicating that *p*-menthane-3,8-diol also degrades with increasing temperature.

For both 2-butyl-2-ethyl-1,3-propanediol and benzyl benzoate, $F^{\text{lack of fit}} < F^{\text{crit}}$ when considering the three different samples and when considering the effect of temperature. Hence, no significant difference between samples 1, 2 and 3 was observed, as expected. These two actives were also found to be stable at increased temperatures and time within each period, as indicated by the smaller F^{crit} values when compared with the $F^{\text{lack of fit}}$ for each variable.

The instability of the PMD was investigated. It was found that the particular sample of PMD used for these stability tests was not neutralized before isolation and contained small amounts of sulphuric acid used as catalyst during the synthesis. Proper neutralization should reduce the tendency of PMD to degrade. This will, however, need to be confirmed in a new series of stability studies, but

this time using the final formulation in its packaging as intended for market release.

CHAPTER 4

CONCLUSION

The results obtained from the various efficacy testing studies at the SABS Test House in Pretoria confirmed that synergism does exist between the three actives *p*-menthane-3,8-diol, benzyl benzoate and 2-butyl-2-ethyl-1,3-propanediol which were then combined in specific ratios to form the final formulation.

The final combination of the actives *p*-menthane-3,8-diol (70 %), benzyl benzoate (15 %) and 2-butyl-2-ethyl-1,3-propanediol (15 %) were made up as a 10 % aerosol sample and results compared favourably with that of the commercial samples containing 19.5 % DEET as active. This shows that, by using multiple actives in an insect repellent product, it is possible to decrease the amount of active used due to the additive effect of the combination. There are a number of advantages to this approach that could give such formulations a competitive edge over existing products. These include:

- A safer product due to the considerably lower levels of actives;
- A more effective, longer lasting product;
- The ability to formulate insect repellent consumer products that were previously not possible due to the lower levels of actives required.

However, before any insect repellent product may be marketed for human use, a product application needs to be submitted to the Medical Control Council for approval as stated in Act 101 of 1965. This stringent and costly control measure prevents many companies from researching alternative insect repellent actives with the view to replacing the existing ones (e.g. DEET, DMP), regardless of the health risks involved in the use of these products. It would be particularly difficult for the PE Technikon, the owner of the intellectual property described in this work, to commercialise the IP in view of its status as a tertiary education institution. This does not mean that the work described here is only of academic interest, and the business proposal (**Part B**) that follows explores possible

avenues for the Technikon from which it may derive commercial benefit from its investment in this research.

Certain sections of **Part B** contain the same information found in **Part A,** this is because when this business proposal is to be presented to the Technikon, **Part A** will not be included.

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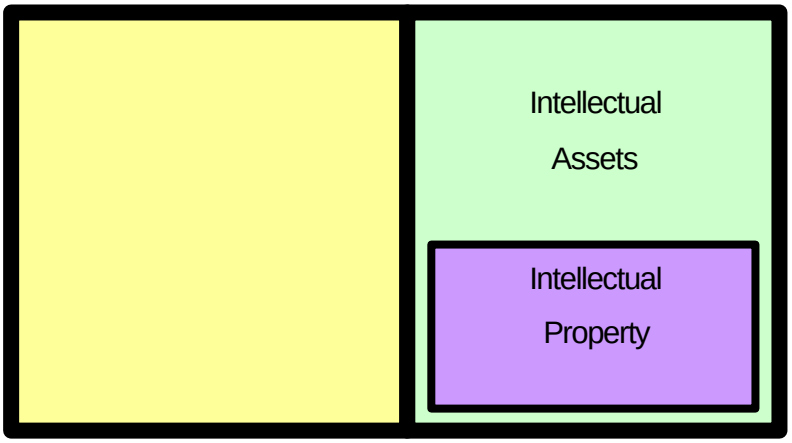
APPENDIX A-LIST OF TABLES

APPENDIX B-LIST OF ILLUSTRATIONS AND FIGURES

PART B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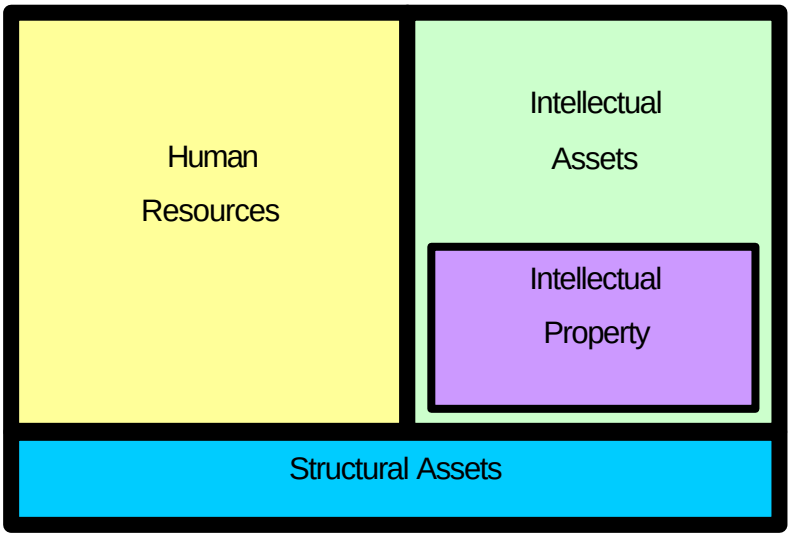
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