

Lure

The invention relates to compositions useful in trapping house dust mites.

- 5 Atopic diseases such as asthma, which are associated with allergens, are a growing public health problem, with the current economic cost of asthma in the UK being estimated in the region of UK £1.05 billion (WHO, 2006). *Dermatophagoides pteronyssinus* (Trouessart) (European house dust mite) and *D. farinae* (Hughes) (American house dust mite) are found throughout the developed world (Fain *et al.*, 1990; Hart, 1995; Arlian and Morgan 2003), and
- 10 are known to excrete allergens that cause atopic diseases (Voorhorst *et al.*, 1964; Tovey *et al.*, 1981; Robinson *et al.*, 1997). A worldwide study concluded that there was a high prevalence of asthma in the UK, as well as other developed countries such as Australia, New Zealand and USA (ISSAC, 1998).
- 15 There are many different approaches to controlling and reducing house dust mite populations: environmentally, by manipulation of habitat conditions, the direct application of chemicals that act by toxic modes of action, allergen removal, and the use of physical barriers such as impermeable bed covers. Relative humidity is known to affect the house dust mite life cycle (Colloff, 1987; Arlian, 1992; Oribe and Miyazaki, 2000). Therefore, by reducing humidity to
- 20 less than 51 %, experimental trials have shown significant reductions in mite and allergen levels (Arlian *et al.*, 2001). Recently, there have been developments on using hygrothermal models to control *D. pteronyssinus* populations (Crowther *et al.*, 2006). Other environmental methods which have undergone experimental trials in an attempt to reduce mite populations include the use of sub-floor heating (de Boer, 2003), steam cleaning (Colloff *et al.*, 1995),
- 25 washing clothes and bed linen above 55°C (McDonald and Tovey, 1992), and freezing soft toys (Nagakura *et al.*, 1996). Synthetic chemical acaricides such as benzyl benzoate and pyrethroids (permethrin and δ -phenothrin), found in commercially available products, can also be used to control house dust mites by applying them to house dust mite habitats (carpets, mattresses, soft furnishings) (Colloff, 1990; Fain *et al.*, 1990; Chang *et al.*, 1996;
- 30 Heide *et al.*, 1997). Permethrin can also be impregnated on to mattress-liners, and a clinical trial demonstrated success at controlling house dust mites for at least 27 months (Cameron and Hill, 2002). Regular vacuum cleaning can reduce the allergen reservoir on beds and carpets, with vacuuming at weekly intervals being more effective than monthly vacuuming (Bellanti *et al.*, 2000). Recommended medical vacuums which are fitted with high efficiency

particulate air (HEPA)-filtering systems that prevent escape of particles >1 µm in diameter reportedly perform better than conventional vacuum cleaners (Colloff *et al.*, 1995). However, the range of efficacy between the HEPA-filtered vacuum cleaners was considerable, with one model performing no better than a conventional vacuum cleaner (Hill and Cameron, 1999). Wet vacuuming might increase the removal of *D. pteronyssinus* allergen, Der p 1, as it is highly water-soluble, although other mite allergens may be less soluble (Colloff *et al.*, 1995). A current more favourable approach is the use of allergen-impermeable bed covers to reduce exposure to allergen in the bed, but studies have shown mixed clinical benefit. For example, one study found a significant reduction in allergen levels, but with no significant improvement in allergic rhinitis in patients (Terreehorst *et al.*, 2003), whereas another study also found a significant reduction in allergen levels with an improvement in asthma symptoms (van den Bemt *et al.*, 2004). However, Luczynska *et al.*, (2003) found that allergen-impermeable covers failed to either reduce allergen levels or alleviate asthma symptoms.

Although there are many methods of house dust mite control, they are not without their limitations. Environmental control measures have to be regularly maintained, otherwise populations will re-establish. Also, if a domestic household receives more knowledge on environmental control, this does not necessarily mean that these measures will be implemented (Callahan *et al.*, 2003). For chemical control, acaricide treatments need to be regularly applied, otherwise re-colonisation of mites will occur, either due to the lack of ovicidal activity (Colloff *et al.*, 1992), or due to a lack of penetration of treatments deep inside the furniture (de Boer, 1998). Furthermore, many asthmatic patients are reluctant to use these products, especially in dormitory areas (Colloff, 1990). Vacuuming needs to occur regularly, and vacuum cleaners cause problems as they can release house dust mite allergen into the air from the vacuum (Kalra *et al.*, 1990). Clinical trials have shown a range of efficacy in using impermeable bed-covers to alleviate symptoms of atopic disease and some patients may find them uncomfortable. Finally, a problem applicable to all control measures is the recruitment of house dust mites from external factors such as dogs (Jackson *et al.*, 2005), car seats (Arlian and Morgan, 2003), hospitals (Custovic *et al.*, 1998) and passenger trains (Uehara *et al.*, 2000).

With current methods exhausted, alternative methods are being studied and tested. For example, incorporating a lure into a house dust mite control method could have the potential to draw mites from deep inside the furniture and mattresses, where upon they would become exposed to a naturally produced acaricide and subsequently vacuumed. Semiochemicals (behaviour-modifying chemicals) have the potential to be used to manipulate house dust mite behaviour, and offer a natural, alternative method to controlling these medically important arthropods. However, the chemical ecology of house dust mites has received little attention, with limited success. Studies have included identifying the chemical profiles of *D. farinae* and *D. pteronyssinus* (Kuwahara *et al.*, 1990; Kuwahara, 1997; Kuwahara, 2004), the identification of the sex pheromone of *D. farinae* (Tatami *et al.*, 2001), and observations on aggregation behaviour but with no chemical identified for evoking the behaviour (Reka *et al.*, 1992; Glass *et al.*, 2001). The inventor has identified house dust mite semiochemicals, and developed a lure-and-kill strategy for mite control.

According to the invention there is provided a composition for luring house dust mites, comprising neryl formate, wherein the composition is in powder form. Alternatively, the composition may be a liquid which forms a powder when it dries.

Also, there is provided a composition for luring house dust mites, comprising limonene, wherein the limonene is substantially in the form of the R-(+)-limonene enantiomer.

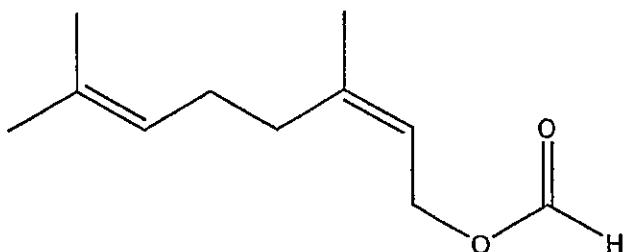
Such compositions may comprise both neryl formate and limonene.

The mite may be any mite, especially a house dust mite or a storage mite.

A house dust mite is an arthropod pest commonly found in the carpets and upholstery of human dwellings. As used herein, the term describes any house dust mite, but particularly refers to *Dermatophagoides pteronyssinus*, the European house dust mite, and *Dermatophagoides farinae*, the American house dust mite.

A storage mite is also an arthropod pest, usually found in stored grains and mouldy environments.

Neryl formate is a semiochemical obtained from house dust mites. It is also known as (Z)-3,7-dimethyl-2,6-octadienyl formate and has the following formula:



5 Limonene is a chiral molecule with two enantiomers R-(+)-limonene and S-(-)-limonene. Racemic limonene (equal amounts of both enantiomers) is known as dipentene. Limonene is a terpene and is well known in the art. The limonene in the composition is preferably substantially in the form of the R-(+)- enantiomer, in other words, preferably at least 65% of the limonene is in the form of the R-(+)- enantiomer, more preferably at least 75%, even
10 more preferably at least 85%, more preferably at least 95%. Most preferably, the composition does not contain S-(-)-limonene. Where the term limonene is used in this specification, it is preferably used to refer to the R-(+)- enantiomer.

The composition is preferably in powder form. This allows it to be easily dusted or shaken
15 over a carpet or item of furniture that may contain dust mites. The dust mites are attracted to the composition and are drawn towards the surface of the carpet or piece of furniture, from where they can be more easily removed. Normally dust mites cling to fibres in, for example, carpets, mattresses and pillows and cannot easily be removed. However, by drawing them to the surface, the dust mites can be more easily removed by vacuuming.

20 It may be preferable in some circumstances for the neryl formate or limonene to be formulated into a powder product which is suitable for sprinkling or dusting. This may be achieved by simple mixing of a small amount of the neryl formate (e.g. a few %w/w) with suitable inert powders, e.g. mineral clay, Fuller's earth, talc, calcium sulphate and/or starch,
25 such that the inert powders do not agglomerate to such an extent as to destroy their sprinklable/dustable properties. Alternatively, the neryl formate may be made into a suspension/emulsion together with an amphipathic carrier (e.g. a modified starch) in an aqueous medium. The suspension/emulsion may then be spray dried, the product of which is a dustable powder containing neryl formate partially or completely encapsulated within the
30 carrier. In many instances, a preferred means of forming a powder formulation is to prepare

granules. These may be prepared, for example, by mixing the neryl formate with a diluent powder (e.g. calcium sulphate, calcium phosphate, calcium carbonate, starch or microcrystalline cellulose (all of which are insoluble, and preferred), or e.g. lactose, mannitol, sucrose, sorbitol or dextrose) and a small amount of binder (e.g. an aqueous, alcoholic or hydroalcoholic solution of cornstarch, gelatin, sucrose, acacia, polyvinylpyrrolidone, methylcellulose, sodium carboxymethylcellulose, polyvinyl alcohol or polyethylene glycol) so as to form a moist mass, coarse screening the mass, drying the resulting moist granules, screening the dried granules and optionally mixing with a glidant (e.g. talc, aerated silica or magnesium stearate).

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In particular, the composition may be produced in powder form by, for example, formulating the neryl formate and/or limonene into a wax and then into particles. In particular it may be formulated into silicate particles.

15 In an alternative formulation, the composition may be in the form of a liquid, especially a solution that is arranged to form a powder when it dries. In particular, the composition may be provided in container provided with a nozzle, such as a misting or aerosol nozzle, through which the composition may be driven. The composition may be driven through the nozzle by a pump or any other appropriate means.

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The composition may further comprise a compound that is toxic to house dust mites, such as an acaricide. An acaricide is an agent used to kill mites. As used herein, the term includes any type of acaricide, such as natural pyrethroids and essential oils. One particular acaricide that may be used is pyrethrum. The use of other acaricides are also envisaged, especially plant based acaricides.

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Also provided by the invention is a composition for luring and killing mites comprising neryl formate and/or limonene and an acaricide.

30 The composition may be in powder or other form. It may additionally comprise appropriate carriers.

The compositions of the invention preferably comprise neryl formate and limonene, especially the R-(+)- enantiomer of limonene.

The compositions according to the invention preferably comprise neryl formate and/or limonene and/or an acaricide in effective amounts. That is to say, the compositions preferably comprise sufficient neryl formate and/or limonene to lure dust mites to the surface of the material to which the composition is to be applied. The compositions preferably comprise sufficient acaricide to kill at least 50% of the dust mites lured to the surface. The compositions preferably comprise at least 0.01% neryl formate, more preferably at least 0.05%, more preferably at least 0.5%, even more preferably at least 1%.

The compositions may also comprise one or more other mite semiochemicals, especially one or more other attractants.

The invention further provides the use of neryl formate and/or limonene as a lure or an attractant. for house dust mites.

A lure or attractant for house dust mites is a composition that attracts house dust mites to it.

Also provided is the use of neryl formate and/or limonene and an acaricide to produce a lure-and-kill composition for house dust mites. A lure-and-kill composition is a composition that attracts house dust mites to it and then kills them. The term lure-and-kill is well known in the art.

Further provided is a method of luring house dust mites comprising exposing the house dust mites to neryl formate and/or limonene.

The term exposing house dust mites to neryl formate and/or limonene preferably means placing neryl formate and/or limonene near to house dust mites. In particular, the neryl formate and/or limonene may be placed within 5cm of the mites, more preferably within 4cm of the mite, even more preferably within 3 cm of the mites.

The method may additionally include the step of exposing the house dust mites to an acaricide. Preferably the house dust mites are exposed to the acaricide simultaneously or subsequently to exposure to the neryl formate and/or limonene.

The invention will now be described in detail, by way of example only, with reference to the following drawings:

Fig. 1: Proportion of *Dermatophagoides farinae* mites caught in the lure area

5 (20 mm centre of carpet disc) over time;

Fig. 2: Proportion of *Dermatophagoides pteronyssinus* mites caught in the lure area

(20 mm centre of carpet disc) over time;

10 Fig. 3: Release rates for the three formulations;

Fig. 4: Total Number of *Dermatophagoides farinae* retrieved in “sprinkle zone” after exposure to 0.05% neryl formate formulation;

15 Fig. 5: GC traces of A) (R)-limonene + *D. pteronyssinus*, B) racemic limonene and C) (R)-limonene; and

Fig. 6: Percentage Mortality Data for *Dermatophagoides farinae* after exposure to pyrethrum

20 Example 1 - Neryl Formate

METHODS AND MATERIALS

House dust mites. *Dermatophagoides farinae* were reared as previously reported (Skelton *et al.*, 2007). *Dermatophagoides pteronyssinus* mites were fed on a mixture of ground yeast cells (Allison) and fish flakes (TetraMin), at 23-25°C and 70-75% relative humidity (Spieksma, 1967; Arlian *et al.*, 1990) and maintained under these conditions until required for behavioural bioassays and chemical analysis.

Preparation of house dust mite extracts. *Dermatophagoides farinae* and *D. pteronyssinus* cultures (0.1 g) were placed separately at the top of glass measuring cylinders (100 ml) containing saturated NaCl solution (80 ml) (Hart and Fain, 1987; Fain and Hart, 1986). After 10-15 min, mites that remained floating were pipetted into a glass vial. Distilled hexane (10 ml) was added and then left at 4°C overnight. The solvent layer of each extract was removed into a clean vial, and dried using anhydrous magnesium sulphate. The extract was filtered

and concentrated under a gentle stream of nitrogen to a volume of either 50 or 100 μ l. The *D. farinae* extract was fractionated by liquid chromatography over Florisil® (60-100 mesh, Aldrich Chemical Company, Gillingham, UK), using distilled hexane (100 %), hexane: diethyl ether (5, 10, 20, 50 %), diethyl ether (100 %) and dichloromethane (100 %) as eluants.

Collection of House Dust Mite Volatiles. *Dermatophagoides farinae* and *pteronyssinus* mites were transferred from a Petri dish into the PTFE tube using a glass pipette tip and gentle suction. Silanized glass wool was compacted at both ends of the PTFE tube and attached to Tenax TA tubes, at one end, and sealed using Swagelok connectors at the other. A control was carried out simultaneously with another PTFE tube compacted with glass wool and either left empty or containing fish flakes. A positive humidified air-flow was introduced into the PTFE tubes at 100 ml/min, through a charcoal filter to reduce contamination, whilst a negative air-flow was drawn through the PTFE tubes simultaneously at 100 ml/min to indicate the air speed through the PTFE tubes. The air entrainment lasted for 24 h after which the Tenax TA tubes were removed.

Gas Chromatography (GC). Mite extracts and chromatography fractions were analysed using a Hewlett Packard 6890 GC equipped with a cross-linked methyl silicone capillary column (50 m, 0.32 mm i.d, 0.52 μ m film thickness) fitted with a cool-on-column injector and a flame ionization detector (FID). The GC oven was programmed to heat from 30°C to 230°C at 10°C/min, and held at 230°C for 30 min. The carrier gas was hydrogen. Tenax TA samples were analysed on a Hewlett Packard 6890 GC machine, equipped with a cross-linked methyl silicone capillary column (50m, 0.32 mm i.d, 0.82 μ m film thickness) and a FID. Thermal desorption occurred inside the programmed temperature vaporisation unit (PTV), which was programmed to heat rapidly from 30°C to 220°C (16°C/sec). The GC oven was then maintained at 30°C for 30 sec, and then programmed at 5°C/min increments to 120°C, followed by 10°C/min to 240°C. The carrier gas was hydrogen.

Gas Chromatography-Mass Spectrometry (GC-MS). Mite extracts and fractions were analysed using a VG AutoSpec mass spectrometer (Fisons Instruments, Manchester, UK), coupled to a Hewlett Packard 5890 GC equipped with a cool-on-column injector. Ionization was by electron impact (70eV, 250°C). The GC oven was programmed to heat at 30°C for 5

min and then 5°C/min until 250°C. The carrier gas was helium. Tentative identifications of mite-specific peaks were based upon comparison of acquired mass spectra with current MS databases (NIST, 2005) or with MS data published in the literature. Identifications were confirmed by peak enhancement with authentic samples (Pickett, 1990).

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Synthesis and Quantification of Neryl Formate. Neryl formate was synthesized in one step from commercially available nerol (> 97% purity by GC, Aldrich Chemical Company, Gillingham, UK) using formic acid and 1,3-dicyclohexylcarbodiimide. To calculate the amount of neryl formate present in mite extracts, a single point external standard
10 quantification method was used (Alltech Association, 1998) using peak area data from acquired GC traces.

House Dust Mite Behaviour. A Y-tube olfactometer was used to observe the behavioural responses elicited by house dust mites, as described previously (Skelton *et al.*, in press). For
15 each experiment, a treatment and control stimulus was added (1 µl) to separate filter paper discs (1.5 cm diameter), allowed to dry for 1 min, and placed into each arm of the olfactometer. A power analysis was carried out at 80 % power, 95 % significance; to calculate appropriate sample sizes to see 40 % difference in effect (70 % to treatment) (STATA 8.2 software). Therefore, 20 mites which made a choice were recorded for each
20 treatment experiment as well as mites who failed to make a choice. Treatments comprised: (1) chromatography fractions derived from the *D. farinae* extract .v. hexane, (2) neryl formate (10 & 100 ng/µl) .v. hexane, (3) a female *D. farinae* extract .v. neryl formate, (4) a male *D. farinae* extract .v. neryl formate, and (5) a female *D. pteronyssinus* extract .v. neryl formate.

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Statistical Analyses. Categorical data from the Y-tube olfactometer bioassays were tested with a Chi-squared test for goodness of fit, with a Yates correction factor (preference to a particular arm) (Fowler *et al.*, 1998). Time data were log 10-transformed prior to parametric data analysis. A three-way ANOVA (Minitab 11 for Windows) was carried out to analyse if
30 the log time to make a decision in the treatment arm (neryl formate) was affected by the concentration of neryl formate, gender and house dust mite species studied.

RESULTS

Confirmation and Quantification of Neryl formate. Coupled GC-MS analysis of the *D. farinae* fractions suggested that neryl formate was found in fraction 4. The identification was confirmed by the co-injection of fraction 4 with an authentic sample of neryl formate on two GC columns of differing polarity. Using a single point external standard quantification method, the amount of neryl formate per *D. farinae* mite was calculated to be 1.32 ± 0.2 ng and 3.3 ± 0.3 ng for males and females respectively. The amount of neryl formate per *D. pteronyssinus* mite was 0.5 ± 0.01 ng and 1.13 ± 0.11 ng for males and females, respectively. Qualitative analysis of the air entrainment samples indicated neryl formate was present in both *Dermatophagoides farinae* and *D. pteronyssinus* extracts.

Behavioural Activity to House Dust Mite Derived Fractions. The global responses (males and females of *D. farinae*) to the treatment arm were only significant when fraction four was tested ($\chi^2 = 4.05$, $df = 1$, $P < 0.05$, $n = 20$). No significant difference was found for the other fractions tested.

House Dust Mite Olfactory Responses to Neryl Formate. The global responses of males and females of *D. farinae* when exposed to neryl formate at 10 ng ($\chi^2 = 9.025$, $df = 1$, $P < 0.01$, $n = 40$) and 100 ng ($\chi^2 = 4.225$, $df = 1$, $P < 0.05$, $n = 40$) were significant to neryl formate (Figure 3). The global responses (males and females of *D. pteronyssinus*) when exposed to neryl formate at 10 ng ($\chi^2 = 11.025$, $df = 1$, $P < 0.01$, $n = 40$) and 100 ng ($\chi^2 = 11.025$, $df = 1$, $P < 0.01$, $n = 40$) were also significant to the arm containing neryl formate (Figure 3). However, there were observed variations in the responses between genders. The males of *D. farinae* only demonstrated a significant response to neryl formate at 100 ng ($\chi^2 = 6.05$, $df = 1$, $P < 0.05$, $n = 20$) and the females demonstrated a significant response only at 10 ng ($\chi^2 = 6.05$, $df = 1$, $P < 0.05$, $n = 20$). Males of *D. pteronyssinus* demonstrated a significant response to neryl formate at 10 ng only ($\chi^2 = 6.05$, $df = 1$, $P < 0.05$, $n = 20$), whereas the females of *D. pteronyssinus* demonstrated a significant response to both 10 ng ($\chi^2 = 4.05$, $df = 1$, $P < 0.05$, $n = 20$), and 100 ng ($\chi^2 = 8.45$, $df = 1$, $P < 0.01$, $n = 20$). There were no significant differences in the log time taken by *D. farinae* and *D. pteronyssinus* to move past the 1 cm mark point along the arm containing neryl formate when analysing concentration (10 ng against 100 ng of neryl formate) ($F = 2.38$, $df = 1$, $P = 0.125$) or gender effects ($F = 1.60$, $df = 1$, $P = 0.208$). However, a significant difference was found between the log time taken by *D. farinae* compared to *D. pteronyssinus* to move past the 1 cm mark point of the arm containing neryl formate ($F = 10.30$, $df = 1$, $P < 0.02$).

House Dust Mite Olfactory Responses to Synthesised Neryl Formate versus Neryl Formate from a Con-Specific Extract. For *D. farinae* males, there was no significant difference in the response to synthetic neryl formate or the male *D. farinae* extract containing naturally produced neryl formate ($\chi^2 = 0.10$, $df = 1$, $P = 0.75$, $n = 14$). Similarly, for *D. farinae* females there was also no significant difference in the response to either neryl formate or the female *D. farinae* extract ($\chi^2 = 1.38$, $df = 1$, $P = 0.24$, $n = 18$) (Figure 3). The responses by *D. pteronyssinus* females to either synthetic neryl formate or the female *D. pteronyssinus* extract were not significantly different ($\chi^2 = 0.06$, $df = 1$, $P = 0.80$, $n = 16$).

DISCUSSION

Dermatophagoides farinae did not demonstrate significant behavioural activity to fractions one, two, three, five and six derived from the *D. farinae* extract, but did respond significantly to fraction four. Neryl formate was tentatively identified in fraction four using GC-MS, and confirmed by peak enhancement, using both a HP-1 and DB wax column. Neryl formate has only been previously tentatively identified as a component of both *D. farinae* and *D. pteronyssinus* extracts (Kuwahara *et al.*, 1990; Tatami *et al.*, 2001) as no peak enhancement was carried out. In the current study, neryl formate elicited significant orientated responses by males and females of *D. farinae* and *D. pteronyssinus* at concentrations of 10ng and 100ng. Other astigmatic mite aggregation pheromones are active at these concentrations: for example *Caloglyphus polyphyllae* mites significantly respond to β -acaridial at 10ng (Shimizu *et al.*, 2001) and *Lardoglyphus kono* significantly responds to (1*R*, 3*R*, 5*R*, 7*R*) lardolure at 10ppm (10ng/ μ l) (Kuwahara *et al.*, 1991). When synthetic neryl formate was tested for behavioural responses against the con-specific extract containing neryl formate at the same level, neither *D. pteronyssinus* nor *D. farinae* showed a significant preference for either arm. The results confirm that the chemical causing the behaviour observed in the initial fraction experiment was elicited by the presence of neryl formate. Data analysis of the time showed *D. pteronyssinus* and *D. farinae* moved along the arm at significantly different times when exposed to neryl formate, but this does not infer *D. pteronyssinus* walked faster, as the track the mite moved along was not measured.

As both species of house dust mite responded to neryl formate, by definition the chemical is not acting as an aggregation pheromone, however, semiochemicals are known to induce similar behaviour in closely related species. For example, neryl formate and citral evoke the

same behavioural response in four species of *Rhizoglyphus* mites (Akiyama *et al.*, 1997). However, the role of neryl formate in house dust mite behaviour remains unclear. *Dermatophagoides farinae* have been observed clustering in the laboratory (Glass *et al.*, 1998; Reka *et al.*, 1992) and both *D. farinae* and *D. pteronyssinus* have been observed
5 clustering together in this study (A. Skelton, personal observation). It has been suggested that the clustering behaviour is initiated by an arrestant-aggregation pheromone in house dust mite faeces, but the study observing this behaviour did not identify the chemical or chemicals involved (Reka *et al.*, 1992). Therefore, the behavioural responses observed to neryl formate, along with the presence of the chemical in house dust mite volatile collections, suggests it
10 may be involved in the clustering behaviour of house dust mites. The ecological benefits of clustering by house dust mites could be to either protect themselves from dehydration, or as a defence mechanism. Mites may cluster together to reduce the surface area that is exposed to the drier environment and subsequently prevent dehydration. There is evidence to suggest that arthropods aggregate to form a “super-organism” to reduce water loss by reducing the
15 surface area of the individual arthropod, and this behaviour has been observed in *Stenotarus rotundus*, the tropical fungus beetle (Yoder *et al.*, 1992). However, males of a *D. farinae* colony have been observed clustering at hydrating conditions (75 % RH). Therefore, temperature and humidity may be ruled out as factors instigating the clustering behavioural response, and semiochemicals suggested (Glass *et al.*, 1998). Alternatively, clustering
20 together may serve as a defence mechanism to protect against potential predators, e.g. Cheyletidae mites (Colloff, 1991) and the formation of big clusters of house dust mites in the homes may disorientate the predators at locating an individual mite (Franz *et al.*, 2001). However, neryl formate may play no role in the clustering of mites. The chemical is commonly found in astigmatic mites (Kuwahara, 2004), and was recently discovered as an
25 aggregation pheromone in *Rhizoglyphus setosus* (Kuwahara, 2006). Neryl formate may be involved in house dust mite recognition of a population presence, with subsequent species-specific semiochemical cues used later to locate a mate, e.g. 2-hydroxy-6-methylbenzaldehyde, which initiates mounting behaviour in male *D. farinae* (Tatami *et al.*, 2001).

30 The lack of behavioural studies on house dust mites, including studies in homes, has contributed to the problem of house dust mite control. However, the current study has identified a means for controlling house dust mite populations, and could ultimately help in alleviating the symptoms associated with atopic diseases.

Example 2 - Neryl Formate*Development of semi-field bioassay for testing semiochemicals*

Initially, a bioassay was designed to test the efficacy of neryl formate in attracting populations of the American house dust mite, *Dermatophagoides farinae*, and the European house dust mite, *D. pteronyssinus*, from carpet sections. The bioassay involved the placement of *D. farinae* and *D. pteronyssinus* onto cut carpet discs (5cm in diameter) which were placed underneath a glass Petri dish to maintain humidity. Two types of carpet (both obtained from Heuga Home Flooring BV) were tested: cut pile (Simply Soft Aquarius) and loop pile (Working Week Boucle Ink Pad). Aliquots of a 10 ng/ 10 µl neryl formate solution (in hexane) were used for the lure treatment, whilst hexane alone was used for the control. Both solutions were injected onto filter paper discs (2 cm diameter) and placed into the centre of the carpet discs. After an allocated time interval (1, 5, 10, 20 or 40 min), a sticky trap was placed onto the carpet to capture the mites, and subsequently placed under a microscope to record the amount and position of the mites, to ascertain if the mites had moved from their original position. Data were analysed using a generalized linear model assuming a binomial distribution with a logit link and dispersion parameter equal to 1.0 (see Table 1). The analysis of deviance for both responses was obtained using the MODEL procedure as implemented in GenStat 8.0 (Rothamsted Research).

Table 1 Accumulated Analysis of Variance

Factor	Total Numbers Retrieved		Target against <i>n</i>		Target against count	
	<i>D. farinae</i>	<i>D. pteronyssinus</i>	<i>D. farinae</i>	<i>D. pteronyssinus</i>	<i>D. farinae</i>	<i>D. pteronyssinus</i>
Carpet	<.001	<.001	<.001	0.001	0.003	0.080
Treatment	<.001	<.001	<.001	<.001	<.001	0.022
Time	0.462	0.514	0.004	<.001	0.007	<.001
Carpet.Treatment	0.053	0.942	0.039	0.673	0.086	0.550
Carpet.Time	0.779	0.290	0.653	0.294	0.858	0.142
Treatment. Time	0.238	0.288	0.105	0.595	0.119	0.836
Carpet.Treatment.Time	0.635	0.907	0.231	0.316	0.265	0.184

Target data corresponds to mites found on the filter paper or in the area where the filter paper was placed. *n*= 40 number of mites per bioassay. Count = numbers retrieved in bioassay

The data suggested that the presence of neryl formate significantly increased the number of mites retrieved on sticky traps, with the lures working more effectively on the cut pile carpet

than the loop pile (see Figs 1 & 2). The practicality of using a physical trap was assessed after these experiments. Consequently, it was proposed that using neryl formate in a slow-release formulation, which could be shaken on to the carpets to lure the mites to the surface, then subsequently removed by vacuum cleaning, would be more feasible and a user-friendly concept.

Development of a slow-release powder formulation for neryl formate

Neryl formate was formulated into a wax, then into silicate particles (100 μm diameter). Three formulations were developed, at 5 %, 0.5 % and 0.05 %. To determine the release rate for neryl formate from each formulation, small amounts (1 mg) were subjected to air entrainment using TENAX TA as adsorbent. Time interval periods for sampling were 1, 20 and 40 minutes. After the allocated time, TENAX TA tubes were removed, and the trapped volatiles were analysed on a Hewlett Packard 6890 GC, equipped with a cross-linked methyl silicone capillary column (50m, 0.25 mm i.d, 0.32 μm film thickness) and a FID. The compound was removed from the tube onto the GC column by thermal desorption, using a PTV unit, which was programmed to heat rapidly from 30°C to 220°C (16°C/s). The GC oven was maintained at 30°C for 30 seconds, then programmed to rise at 5°C/min to 120°C, followed by 10°C/min to 240°C. The carrier gas was hydrogen. Release rates for the three formulations are shown below (Fig 3).

The neryl formate formulation at 0.05 % was selected for further work. Twenty and forty minutes were chosen as the two time points to test the formulation, compared against a blank formulation, using the bioassay described above.

Table 2 Accumulated Analysis of Variance

Factor	Total Numbers Retrieved	Numbers in "Sprinkle Zone"
Treatment	0.004	<.001
Time	0.066	0.052
Treatment.Time	0.966	0.557

"sprinkle zone" = the area of carpet covered by the powder.

The data suggested that at 20 minutes, significantly more *D. farinae* mites were retrieved in the sprinkle zone than at 40 minutes (see Fig. 4).

Example 3 - Limonene

5 *House dust mites.*

Dermatophagoides pteronyssinus mites were fed on a mixture of ground yeast cells (Allison) and fish flakes (TetraMin), at 23-25°C and 70-75% relative humidity (Spieksma, 1967; Arlian *et al.*, 1990) and maintained under these conditions until required for behavioural bioassays and chemical analysis.

10

Preparation of house dust mite extracts.

D. pteronyssinus cultures (0.1 g) were placed separately at the top of glass measuring cylinders (100 ml) containing saturated NaCl solution (80 ml) (Hart and Fain, 1987; Fain and Hart, 1986). After 10-15 min, mites that remained floating were pipetted into a glass vial.

15

Distilled hexane (10 ml) was added and then left at 4°C overnight. The solvent layer of each extract was removed into a clean vial, and dried using anhydrous magnesium sulphate. The extract was filtered and concentrated under a gentle stream of nitrogen to a volume of either 50 or 100 µl. The *D. farinae* extract was fractionated by liquid chromatography over Florisil® (60-100 mesh, Aldrich Chemical Company, Gillingham, UK), using distilled
20 hexane (100 %), hexane: diethyl ether (5, 10, 20, 50 %), diethyl ether (100 %) and dichloromethane (100 %) as eluants.

Collection of House Dust Mite Volatiles.

Dermatophagoides pteronyssinus mites were transferred from a Petri dish into the PTFE tube
25 using a glass pipette tip and gentle suction. Silanized glass wool was compacted at both ends of the PTFE tube and attached to Tenax TA tubes, at one end, and sealed using Swagelok connectors at the other. A control was carried out simultaneously with another PTFE tube compacted with glass wool and either left empty or containing fish flakes. A positive humidified air-flow was introduced into the PTFE tubes at 100 ml/min, through a charcoal
30 filter to reduce contamination, whilst a negative air-flow was drawn through the PTFE tubes simultaneously at 100 ml/min to indicate the air speed through the PTFE tubes. The air entrainment lasted for 24 h after which the Tenax TA tubes were removed.

Gas Chromatography (GC).

Mite extracts and chromatography fractions were analysed using a Hewlett Packard 6890 GC equipped with a cross-linked methyl silicone capillary column (50 m, 0.32 mm i.d, 0.52 μ m film thickness) fitted with a cool-on-column injector and a flame ionization detector (FID). The GC oven was programmed to heat from 30°C to 230°C at 10°C/min, and held at 230°C for 30 min. The carrier gas was hydrogen. Tenax TA samples were analysed on a Hewlett Packard 6890 GC machine, equipped with a cross-linked methyl silicone capillary column (50m, 0.32 mm i.d, 0.82 μ m film thickness) and a FID. Thermal desorption occurred inside the programmed temperature vaporisation unit (PTV), which was programmed to heat rapidly from 30°C to 220°C (16°C/sec). The GC oven was then maintained at 30°C for 30 sec, and then programmed at 5°C/min increments to 120°C, followed by 10°C/min to 240°C. The carrier gas was hydrogen.

Gas Chromatography-Mass Spectrometry (GC-MS).

Mite extracts and fractions were analysed using a VG AutoSpec mass spectrometer (Fisons Instruments, Manchester, UK), coupled to a Hewlett Packard 5890 GC equipped with a cool-on-column injector. Ionization was by electron impact (70eV, 250°C). The GC oven was programmed to heat at 30°C for 5 min and then 5°C/min until 250°C. The carrier gas was helium. Tentative identifications of mite-specific peaks were based upon comparison of acquired mass spectra with current MS databases (NIST, 2005) or with MS data published in the literature. Identifications were confirmed by peak enhancement with authentic samples (Pickett, 1990).

Confirmation of Limonene in Dermatophagoides pteronyssinus extracts

Limonene was tentatively identified in *D. pteronyssinus* extracts by GC-MS but required confirmation using peak enhancement.

Materials and Methods

Equipment

R (+) Limonene 97 % (Sigma Aldrich)
S (-) Limonene 98 % (Sigma Aldrich)
D. pteronyssinus extract

Procedure

The racemic mixture of limonene was analyzed by GC on a β -cyclodextrin chiral capillary column (30 m x 0.25 mm ID x 0.25 μ m film thickness) using a HP5890 GC (Agilent Technologies, UK) fitted with a cool-on-column injector and a FID. The GC oven temperature was maintained at 30°C for 1 min after sample injection and then raised by
5 0.5°C/ min to 40°C where it was held for 20 min. The carrier gas was hydrogen.

Results

As shown in the graph in figure 1, R-(+)-limonene was the enantiomer present in the *D. pteronyssinus* extracts. R-(+)-Limonene was also found in extracts of the chicken mite,
10 *Dermanyssus gallinae*.

R-(+)-Limonene was specific to *D. pteronyssinus* and identified in both solvent and air entrainment extracts, and was not identified in any *D. farinae* extracts.

Example 4 - Acaricides

15 Identification of potential acaricide to be incorporated into lure-and-kill system

Natural pyrethrum, known for its insecticidal properties, and azadirachtin, the active ingredient of neem, were tested for their lethal dosage (LD) using an established bioassay. Pyrethrum was more effective than azadirachtin, where little mortality was seen. Pyrethrum LD₅₀ values at 1, 2, 3 hours post exposure were 384.53 ppm, 20.829 ppm and 0.219 ppm,
20 respectively (see Fig. 6). The LD₉₀ values for pyrethrum 2 and 3 hours post exposure were 2663.9 ppm and 225.86 ppm respectively.

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CLAIMS

1. A composition for luring mites, comprising neryl formate, wherein the composition is in powder form or forms a powder on drying and further comprises a compound that is toxic to mites and wherein the mites are house dust mites.
2. A composition for luring and killing mites comprising neryl formate and an acaricide, wherein the mites are house dust mites.
3. A composition for luring and killing mites according to claim 2 further comprising limonene wherein the limonene is in the form of the R-(+)-limonene enantiomer and wherein the mites are house dust mites.
4. A composition according to any preceding claim, comprising one or more additional attractants.
5. A kit for luring and killing mites comprising a supply of neryl formate and a supply of an acaricide, wherein the mites are house dust mites.
6. A kit according to claim 5 further comprising a supply of limonene wherein the limonene is in the form of the R-(+)-limonene enantiomer.
7. The use of neryl formate as a lure or an attractant for mites, wherein the mites are house dust mites.
8. The use of neryl formate and limonene as a lure or an attractant for mites wherein the limonene is in the form of the R-(+)-limonene enantiomer, wherein the mites are house dust mites.
9. The use of neryl formate and an acaricide to produce a lure-and-kill composition for mites, wherein the mites are house dust mites.
10. The use of neryl formate and limonene and an acaricide to produce a lure-and-kill composition for mites wherein the limonene is in the form of the R-(+)-limonene enantiomer, wherein the mites are house dust mites.

11. A method of luring house dust mites comprising exposing the house dust mites to neryl formate.
12. A method of luring house dust mites comprising exposing the house dust mites to neryl formate and limonene wherein the limonene is in the form of the R-(+)-limonene enantiomer.
13. The method of claim 11 or 12, further comprising the step of exposing the house dust mites to an acaricide.