Insect Repellents Based on *para*-Menthane-3,8-diol

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To my parents, thank you
ever so much for your generous
support and patience

Abstract

Vector-borne diseases transmitted by mosquitoes are a major cause of morbidity and mortality in the world by transmitting infections such as malaria, dengue, yellow fever, West Nile fever or chikungunya, to more than 700 million people each year. Without existing vaccines for each disease, skin repellents are one of the most effective defenses against mosquitoes for an individual protection. *N,N*-diethyl-*m*-methylbenzamide (DEET) is an efficient and commonly used active since decades. New molecules have appeared on the market and proved their repellent action mostly comparable to DEET in regards to different vectors, and *para*-Menthane-3,8-diol (PMD) is one of them. In this study, PMD was first synthesized in a new green process and confirmed its high repellent activity against *Aedes aegypti* mosquitoes (dengue, yellow fever, chikungunya, zika vector). The work then consisted to develop formulation systems (surfactantless or classical microemulsions, O/W lotions), which are able to extend the lasting protection of PMD based repellents. As an example, a leave-on lotion was designed with ingredients meeting eco-friendly standards and showed a repellent activity statistically comparable to the reference product on the market, Autan® Protection Plus, with more than 7 hours of protection. Additionally, a large study on the complexation of PMD with cyclodextrins (CD) was considered in order to increase its solubility in water and provide a controlled release over time. PMD:HP-β-CD was confirmed by spectroscopic analysis nevertheless, mosquito repellent tests with *Aedes aegypti* did not show any efficient protection. In a second study, a new ester derived from PMD, so called PMD-Succinate (PMD-S) was investigated and synthesized under sustainable routes. Physical, chemical and cytotoxicity properties are reported and showed as main interests: 1/ a high water solubility whereas the other actives are insoluble, 2/ a surface activity comparable to common hydrotropes in order to solubilize other repellent actives in aqueous mediums

and thus reach longer protection times 3/ a very good tolerance in regards to skin irritations and allergic reactions. These functions highlight the interest for the new PMD-Succinate active in order to make simple, long-lasting, environmentally friendly and harmless formulations to protect the populations against mosquitoes.

In a third part, a study of 19 Corsican (French island) essential oils was carried out in order to determine their protection activity against mosquitoes. *Laurus nobilis* was found to provide the highest repellent action and the preferred hedonic dimension and acceptance by the volunteers. In a near future, this essential oil could be integrated into new repellent formulations based on PMD-S as a booster effect and as a pleasant olfactive signature.

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Chapter 1

GENERAL INTRODUCTION

1.1. Mosquitoes: Vectors of Diseases

1.1.1. General

Mosquitoes (Diptera: Culicidae) are haematophagous insects known worldwide as a biting nuisance, and also for their role in spreading diseases. Pathogens carried by mosquitoes cause diseases including malaria, West Nile fever or neuroinvasive disease, filariasis, yellow fever and dengue fever. The pathogens are transmitted between the mosquito and the host when the mosquito bites. Once a mosquito feeds from an infected host, the pathogens move from the mosquito's gut to the salivary glands, ready for transmission. As the mosquito probes with its proboscis, any pathogens present in the salivary glands are injected into the host. Specific mosquito species transmit specific diseases, because the pathogens ability to complete its lifecycle, by migrating through the gut wall to the salivary glands, is host dependant. There are approximately 3400 known mosquito species, which vary widely in their habit and host range. Some mosquitoes are anthropophilic, feeding only on human beings, some are zoophilic, preferring animals, and some are opportunistic or generalist and feed on many different species[1]. Mosquitoes that bite human beings are of the greatest concern to public health, and these are mostly from the *Anopheles*, *Aedes* and *Culex* Genera[2]. Opportunistic and generalist mosquitoes, which feed on both human beings and other species, pose another problem to the control of diseases, as animals can act as reservoir hosts and maintain the disease even if it is being controlled in the human population. Opportunistic feeding on animals and human beings can also lead to further geographical spread of diseases. An example of this can

be seen with the West Nile virus, which first emerged in North America in 1999 and spread quickly through the United States, Canada, Mexico and the Caribbean basin[3,4]. The spread of the virus to Central and South America was due to the mosquito species, which transmit the disease, Culex pipiens and Culex quinquefasciatus, feeding on both human and bird hosts. Mosquitoes carrying the pathogen passed it to avian hosts, which after migrating long distances, transmitted the disease to mosquitoes in new locations, which then infected human beings[5]. However, most of the spread of vector-borne diseases is due to migration of the mosquitoes themselves as they are accidentally moved by human transportation[6], or by infected humans travelling to an area where there is a vector mosquito population capable of transmitting that disease.

The most well-known and thoroughly studied mosquitoes are of the *Anopheles* Genus, which are the vectors of malaria. However, mosquitoes in the *Aedes* Genus are also extremely important, being responsible for the transmission of a range of diseases including the West Nile, yellow fever, dengue fever and chikungunya viruses. There are over 700 species of *Aedes* mosquitoes, with *Aedes albopictus*, the Asian tiger mosquito, and *Aedes aegypti*, the yellow fever mosquito, the most commonly known. *Aedes aegypti* feeds on both human and animal hosts and is present in urban areas of Africa, South America, Australia and Asia, vectoring the yellow fever, dengue fever and chikungunya viruses. Yellow fever is an acute viral haemorrhagic fever caused by a virus from the Flaviviridae family, with symptoms including fever, nausea, pain and, in some cases, liver damage which can lead to death. There are an estimated 200 000 cases of yellow fever, with 30 000-60 000 deaths, each year. However, significant progress in combatting the disease has been made in West Africa for example and more than 105 million people have been vaccinated in mass campaigns[7].

As mosquitoes have such an important impact upon public health, there is a great interest in studying their biology, genetics and ecology to improve and develop methods of control. An area of particular interest is investigating on how mosquitoes locate their hosts and determining if the process can be interrupted. Some repellents, including the widely used DEET (*N,N*-diethyl-*m*-methylbenzamide), have been thought to work by interrupting the host-seeking process and preventing the mosquito from detecting attractive odors[8,9]. However, different repellents work in different ways, and the modes of action of repellents are not fully understood[10].

1.1.2. Dengue and Chikungunya cases

Dengue

Dengue fever is a febrile disease caused by four virus serotypes of the genus Flavivirus (Flaviviridae), with symptoms of muscle, joint and retro-orbital pain, fever and a rash. One recent (2013) estimate indicates that 390 million dengue infections occur every year (95% credible interval 284–528 million), of which, 96 million (67–136 million) manifest clinically[11]. 3.9 billion people in 128 countries are at risk of infection with dengue viruses[12].

The virus is transmitted to humans by mosquitoes of the genus *Aedes* especially *Aedes Aegypti*, which is the main vector[13]. This disease predominantly occurs in tropical and subtropical regions but recently also in safe areas. For example, in 2010, several cases from native population were reported in North America (Florida) and also in Europe. Especially in metropolitan France where *Aedes Albopictus*, another dengue vector is now implanted[13]. In general, transmission has mostly progressed in urban and peri-urban areas, where the *Aedes* mosquito is particularly active. There is currently no cure and no vaccine. Therapeutic management is based on symptomatic monitoring and treatment.

Chikungunya

Chikungunya is an arthropod-borne virus (family Togaviridae, genus Alphavirus). It is also transmitted by the yellow fever mosquito *Aedes aegypti*. In recent years, Chikungunya switched to an alternative vector, the Asian tiger mosquito *Aedes albopictus*, a vector that has seen a dramatic global expansion in its geographic distribution in the last decade. This resulted in a large epidemic on the Réunion Island (France) in 2005/2006, with an estimated 270 000 cases of infection[14]. In recent years (2004-2011), Chikungunya produced the largest epidemic recorded for an alphavirus, with an estimated 1.4 to 6 million patients, and imported cases reported in nearly 40 countries including Japan and the USA. The first infections in Europe (Italy in 2007 and France in 2010) were also seen during this epidemic[15]. Chikungunya fever usually develops 2-6 days post the infective mosquito bite and results in 95% of all cases in a severe and sudden onset of high fever (>38.9 °C), which poorly responds to antipyretic medication. Fever is often accompanied by myalgia, headache, fatigue, abrupt febrile illness, maculopapular rash and arthritic disease[16]. The name “chikungunya” is derived

from the Makonde language, meaning “that which bends up”, referring to the cramped posture of infected individuals, caused by the severe arthralgia[17]. Although the clinical disease is usually self-limiting, arthritic symptoms may persist from months, to several years[18]. More severe clinical complications involve encephalopathy, hemorrhagic fever, neurological failure and even death in patients with underlying medical conditions. Therapeutic management is based on symptomatic monitoring and treatment.

1.2. Mosquito

1.2.1. Lifecycle

Mosquitoes occupy multiple habitats during their life cycle, with aquatic larval and pupal stages after hatching from the egg, and a non-aquatic adult stage during which they will bite hosts and may transmit pathogens that cause diseases (see Fig. 1.1).

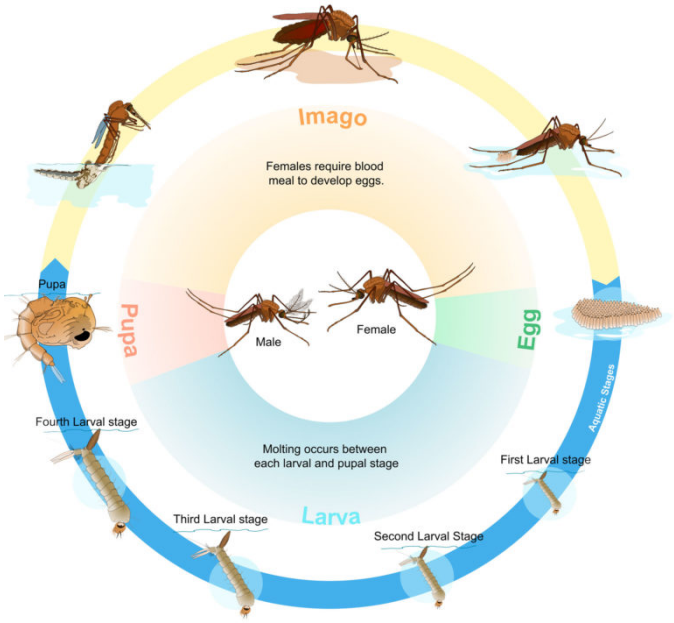


Fig. 1.1: A mosquito life cycle.

Aedes aegypti lays its eggs on damp substrate, just above the water line, around pools of water, which are likely to flood[19]. This can be in disused containers and discarded tires in urban settings, or around ponds and in tree holes in the countryside[2]. The eggs can remain dry for months and still be viable when submerged, making it hard to guarantee

mosquitoes are eliminated from an area and heightening the risk of them being transported between locations in containers or car tires. When the water level rises to cover the eggs, the presence of bacteria in the deoxygenated water stimulates the eggs to hatch and larvae emerge. Various environmental conditions may cause hatching to be delayed (including photoperiods, temperature and the amount of food available), which staggers the emergence of larvae and keeps some eggs in reserve as a survival strategy.

There are four aquatic larval instar stages, which feed on plant and animal micro-organisms in the water and are frequently predated by larger aquatic organisms. Depending on the amount of food available, larvae transform to pupae within 10 days. At the end of the fourth stage, larvae become pupae, mostly staying at the surface of the water and no longer consuming food. After 1-3 days as a pupa, the fully formed adult mosquito emerges from the pupal casing. During the first 24 h as an adult, mosquitoes will seek a sugar meal in order to sustain themselves. Males will feed only on nectar for their entire lifespan, whereas females require a blood-meal in order to develop viable eggs. Females only need to mate once to attain enough spermatozoa for their lifetime of reproduction. In the first two days after emergence, successful mating will take place and females will begin to search for a host, with different mosquito species varying in the time of day when they are most actively host seeking. *Aedes aegypti* is most active at dusk and dawn, but will also bite during the day. It is an endophilic species, naturally associating with humans, and also endophagic, willing to enter houses to bite its hosts. Once the mosquito has located a suitable host, it will attempt to feed by inserting its proboscis into the skin and withdrawing blood. If this process is interrupted, the female will return and make multiple attempts to feed until enough blood has been obtained to produce the eggs. Over the next 2-3 days, the mosquito becomes gravid with developing eggs, and maintains a less active lifestyle. At the end of this time the mosquito will oviposit at a suitable site, possibly a site with a similar odor profile to where it emerged[20]. A female mosquito can lay between 30-300 eggs, with the size of the blood-meal affecting the number of eggs laid[21].

1.2.2. Host

Mosquitoes locate their hosts using heat, moisture, visual and olfactory cues[22]. For the

latter, the volatile chemicals emitting from hosts are detected by the mosquito's antennae and maxillary palps[23]. Such chemicals, which alter the behaviour of the insect when detected, are called semiochemicals[24,25], and the chemicals used by mosquitoes to find their host are kairomones, i.e. compounds which benefit the receiver (the mosquito) to the detriment of the organism releasing the compound (the human host). The ability of a mosquito to detect kairomones from hosts has a direct effect on its ability to transmit the pathogens that cause diseases[26,27]. Several semiochemicals that are released by vertebrates and attract mosquitoes have been identified and one of the most important, which is correlated with mosquito flight activity, is carbon dioxide (CO_2)[28,29]. However, CO_2 from breath accounts for only 50% of the attraction to hosts in highly anthropophilic species[30], indicating that other chemical cues from the host must play an important role in olfactory host-seeking behaviour[31]. *Aedes aegypti* are attracted to volatile chemicals given off in odors from human skin[32-34]. The human body releases around 350 volatile compounds[35] and several of these have been found to be attractive to mosquitoes[36-38]. They show preferences for certain individual human hosts, which may be due to variation in the ratios of these attractive chemicals[38-40]. For some of the chemicals found in human sweat, for example geranyl acetone, 6-methyl-5-hepten-2-one, octanal, lactic acid, nonanal and decanal, people with greater levels than normal are repellent to mosquitoes, and these human-derived compounds can be used as mosquito repellents[38]. One of the key ways to control insects is to manipulate their host-seeking abilities, either by interrupting their detection of attractants, or by causing direct repellency. However, much about the way mosquitoes detect, analyze and act upon chemical cues is still unknown, and this is an important subject to investigate further.

1.3. Mosquito Control

1.3.1. General

There are many intervention strategies used to try to control the spread of diseases by mosquitoes. These mainly fall into the categories of chemical control, environmental control, genetic control and personal protection such as the use of repellents.

Chemical control of mosquitoes encompasses the use of insecticides and larvicides to kill the adults and larvae. For most mosquitoes, larvicides are the most commonly used form of control[19], eliminating the insects before they mature and are able to transmit diseases or reproduce. The most commonly used larvicides are organophosphates and carbamates. However, people are reluctant to contaminate drinking water with chemicals, and environmentally friendly alternatives such as growth regulators are comparatively expensive. Additionally, larvicides have not yet been fully developed and utilized for all mosquito species[41]. There are a wide range of insecticides available to target adult mosquitoes, including organochlorine compounds (such as dichlorodiphenyltrichloroethane, DDT), organophosphates, carbamates, pyrethroids, neonicotinoids and biological pesticides. Most of the non-biological pesticides are highly toxic to wildlife and persist in the environment, thus, the only pesticides sanctioned for use by the World Health Organization are pyrethroids, which have low mammalian toxicity and low persistence[42]. The concentrations of pyrethroids used in household applications also show irritancy and repellent properties, even in pyrethroid-resistant strains, but little is known of the mechanisms responsible for these behaviours[43-45]. The use of pyrethroid-treated bednets is widespread, as these ensure mosquitoes cannot reach the person inside. Even damaged nets give protection as they kill mosquitoes on contact. Although the use of insecticides, and insecticide-treated bed (ITNs), has been very successful for mosquito control, widespread pyrethroid resistance has developed and there are many areas where these insecticides are no longer effective[46]. Environmental control of mosquitoes includes the removal or monitoring of containers or disused tires, which would serve as oviposition sites and the alteration of suitable mosquito habitats such as marshes or ponds by draining them, introducing predatory fish, or increasing water flow so that static pools cannot form. Problems with environmental control include difficulties in maintenance, requiring health education and good communication with local people[47], and the fact that alteration of local habitat may lead to an undesirable rise in abundance of different mosquito species.

There are many studies on the genetic manipulation of mosquitoes, for example by altering their lifespan so that they are less likely to live long enough to transmit malaria[48]. The most common method of genetic control is the release of sterilized males into the wild, which will mate with females but not fertilize their eggs[49]. This technique has only

worked effectively alone in highly isolated areas such as islands, probably because of high genetic variability[50] and the migration of insects[51]. Thus, the high expense and the difficulty in producing enough males to compete with wild mosquitoes (especially as they can have a lower fitness) makes it unpractical as a stand-alone control method, and it is often recommended as part of an integrated control strategy alongside other control measures[52].

1.3.2. Repellents

Integrated control, combining several of the above methods, is considered the most effective way of controlling mosquito populations[19]. An important component of this is personal protection, such as the use of screens, bed nets and repellents, to protect against mosquito bites. The use of ITNs and plant-derived repellents during the hours of greatest mosquito activity has been shown to decrease malaria transmission by up to 80%[53], demonstrating that these methods can be effective against diseases.

Although repellents are difficult to distribute to poor households in rural areas[54], they are considered the first line of defense against mosquitoes[55], preventing biting and therefore stopping transmission of pathogens. There is a wide range of repellents available on the market, of varying levels of effectiveness and which last for different lengths of time. The main active ingredients used in the production of most commercially available repellents are: DEET (*N,N*-diethyl-*m*-methylbenzamide), IR3535 (ethyl butylacetylaminopropionate) and KBR 3023 (1- piperidinecarboxylic acid, 2-(2-hydroxyethyl), 1-methylpropyl ester), which are synthetic compounds, and citronella and *para*-Menthane-3,8-diol (PMD), which are naturally derived from plant oils.

Synthetic repellents may be undesirable to the public, both because they are commonly more expensive than natural alternatives and also due to the smell and greasy feeling when applied to the skin. There are many natural, plant-derived compounds such as citronella oil, thyme oil, and eucalyptus oil which show repellency against mosquitoes[56-60]. However, most plant-derived repellents generally give less protection, or protect for a shorter time, than the minimum 5-6 hours of protection provided by DEET[56,61]. The active ingredient of the most effective natural insect repellent is PMD, derived from lemon eucalyptus oil, which is the only naturally based insect repellent recommended by the

Centre of Disease Control (CDC) for protection against mosquitoes carrying West Nile virus. PMD was found to show an efficacy similar to DEET when DEET was tested at 15 wt.% in ethanol[62-64], but may not provide the same length of protection at higher concentrations of DEET[65].

1.4. *Para*-Menthane-3,8-diol

1.4.1. Overview of PMD

History

In the late 1980's, a worldwide survey was conducted by the U.S. military to identify repellents produced outside the United States. Of the 65 formulations listed, 33 contained DEET and the remainder contained natural oils or undisclosed ingredients. One of the natural compounds identified was quwenling. This formula sparked the interest of researchers because it is a popular repellent in China, where it has been used as insect repellent for more than two decades[66-68]. Quwenling used as natural insect repellent can be obtained from distilling the waste after extraction of *lemon eucalyptus oil*; the main ingredient in this distillate is *para*-Menthane-3.8-diol[69]. While PMD is widely used in China, products containing PMD are relatively new to the U.S. market; hence information on the efficacy of products containing *para*-Menthane-3.8-diol or *para*-Menthane-3.8-diol-rich oils is relatively scarce. This will likely change over time as PMD is more widely used and evaluated in the United States and other developed countries.

Properties

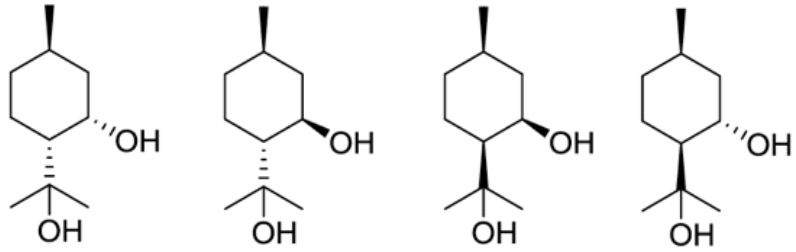
para-Menthane-3.8-diol is a colorless solid, that can either be extracted from the leaves and twigs of Eucalyptus plants, or synthesized commercially as described in this work. Commercial synthesis produces a similar and functionally identical mixture of stereochemical isomers, although it is possible to produce the separate stereoisomers[70](See Scheme 1.1). The following is a summary of the most important chemical and physical properties of PMD (see Table 1.1).

IUPAC Name: 2-(1-hydroxy-1-méthyléthyl)-5-méthylcyclohexanol

CAS Registry number: 42822-86-6

Molecular formula: $C_{10}H_{20}O_2$

Molar Mass: 172.2646 ± 0.01 g/mol



Scheme 1.1: Chemical structure of (±) -cis and (±)-trans *para*-Menthane-3,8-diol isomers.

Property	Result
Colour	Opaque white
Physical State	Solid
Door	Faint mint
Melting Point	34.5°C
Density	0.989 g/ml at 24°C
Solubility	0.29 g/L at 25°C
Vapour Pressure	0.181 Pa, determined by gas saturation method
Dissociation Constant in Water	Not reported; product is not dispersible in water
Octanol/Water Partition Coefficient	Not required, intended use pattern not an environmental fate concern
pH	Not applicable; not dispersible with water
Stability	Stable to sunlight, heat (54°C), metal (iron, aluminium), and metal ions (iron (II) acetate, aluminium acetate)
Flammability	Flash point 139.8°C
Viscosity	56.1 cP at 60°C

Table 1.1: Physical and chemical properties of *para*-Menthane- 3,8-diol.

Toxicity

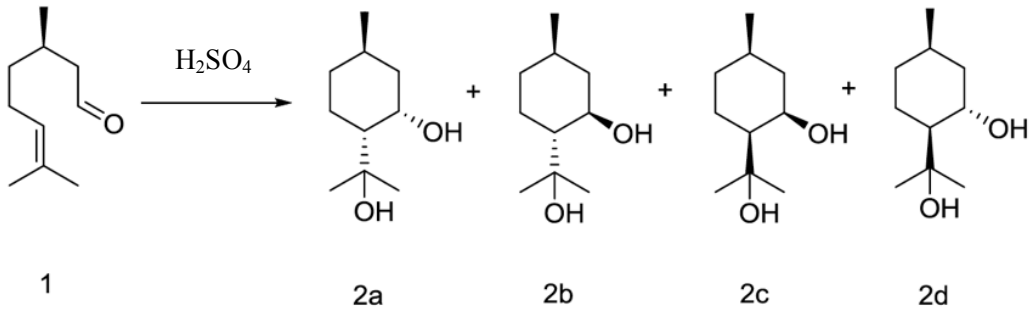
The technical grade active ingredient, *para*-Menthane-3,8-diol, is placed into Toxicity Category IV[71,72] for acute oral toxicity, dermal toxicity and skin irritation, and Toxicity Category I for eye irritation (Toxicity Category II for the end-use product). It is not a skin sensitizer. The no-observed-adverse-effect level (NOAEL) from a 90-day dermal toxicity study in rats was established at a limit dose of 1000 mg/kg/day. The NOAEL for immune suppression, as determined in a 28-day dermal study, via a primary antibody response to sheep red blood cells/plaque forming cell assay was > 3000 mg/kg/day in mice. The NOAEL for maternal and developmental toxicity was established in rabbits at 3000 mg/kg/day by the dermal route. Mutagenicity studies evaluated *para*-Menthane-3,8-diol for its potential to cause point mutations in bacteria and mammalian cells, chromosomal aberrations in mammalian cells, and induction of micronuclei in polychromatic erythrocytes from mouse bone marrow, and found no genotoxicity at the doses tested, with and without metabolic activation. Based on the evaluation of the submitted data, there were no endpoints of concern.

1.4.2. Isolation of PMD

Para-Menthane-3,8-diol is one of a series of naturally occurring compounds obtained from the leaves of *Corymbia citriodora* trees. Eucalyptus ssp. is a genus of trees and shrubs in the family Myrtaceae, which is from Australia, but now commonly grows in almost all tropical and subtropical areas. *Eucalyptus citriodora* leaves contain many compounds with pesticide activity including: aromadendrene, citronellal, citronellic acid, citronellol, citronellyl acetate, *p*-Cymene, limonene, linalool, alpha-pinene, *para*-Menthane-3,8-diol, tannin, terpinene, terpinolene, and ursolic acid. Of the compounds contained in the leave-extracts of *Eucalyptus citriodora*, *para*-Menthane-3,8-diols have been identified as the most noticeable insect repellent. It is also of interest to note that the leave extracts of *Eucalyptus citriodora* also contains citronellol, which is the active ingredient in oil of citronella products but which has a rather poor efficiency in repelling biting mosquitoes[73]. When refined, the oil known as oil of lemon Eucalyptus or, under its trade name, Citriodiol® is used as insect repellent. Typically, Citriodiol contains 64% PMD (a mixture of the cis and trans isomers 62/38 of *para*-Menthane-3,8-diol).

1.4.3. Synthesis of PMD

The PMD is mainly obtained by cyclization of citronellal in an acidic medium (Prins reaction) (see Scheme 1.2). This synthesis is well-known in the literature and has been described with inorganic acids[74-76] or organic acids[77]. The main results are summarized in Table 1.2. and show that PMD is obtained with good yields. In all cases, the stereoisomers **2a** and **2b** are mainly obtained with a higher proportion of the **2a** isomer.



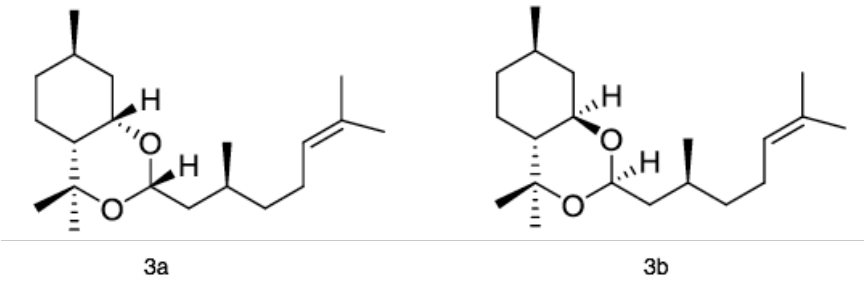
Scheme 1.2: Acid catalyzed synthesis of *para*-Menthane-3,8-diol from citronellal.

The synthesis of *para*-Menthane-3,8-diol has been widely studied with sulfuric acid as a catalyst (Table 1.2, Entries 1-9). Conversion rates of citronellal are above 97%. The acid concentration needed for the synthesis increases as a function of the decrease of the temperature and the reaction time (Table 1.2, Entries 1-5). H_2SO_4 at 0.25% w/w is recommended to achieve a complete conversion of citronellal at 55-60°C as shown with Entries 3 and 4. In all cases, PMD is the main product obtained. The proportion of side products with low boiling points (mainly Isopulegol and Neoisopulegol) increases with temperature (16.5% at 100°C, see Table 1.2, Entry 5). For the reactions conducted at 60°C or below, the main side products are the two acetals **3a-b** represented in Scheme 1.3. They result from the reaction of the intermediate carbocation or the *in situ* PMD formed from the citronellal[75]. The selectivity of PMD depends on the initial concentration of H_2SO_4. The selectivity increases with less sulfuric acid in the medium (see Table 1.2, Entry 1-5). A sulfuric acid concentration of 0.25% w/w leads to a very good selectivity (>90%, Entries 3-4) at 50°C or 60°C with only 2% acetals. The diastereomers **2a** and **2b** are mainly obtained with ratios of isolated products 2a/2b/2c 2d of 64.7/34/0.2/1.1 (see Table 1.2, Entry 4).

Entry	Cat.	Conc.	Time (H)	pH	T (°C)	Conv. %	s/s products	Selectivity %		Yield[f] %	Ref.
								PMD cis	PMD trans		
1	H_2SO_4	10% m	4	-	35	97.1	5.9[a]/20.3[e]	73.8 (64/36)		63	[75]
2	H_2SO_4	3% m	6	-	35	98.3	6.3[a]/14.9[e]	78.8 (64/36)		69	[75]
3	H_2SO_4	0.25% m	11	-	50	97.9	5[a]/2.7[e]	92.3 (64/36)		79	[75]
4	H_2SO_4	0.25% m	7	-	60	98.2	5.7[a]/2.8[e]	91.5 (64/36)		80	[75]
5	H_2SO_4	0.01% m	20	-	100	97.5	16.5[a]/2.7[e]	80.8 (64/36)		70	[75]
6	Acetate buffer[b]	-	4 days	5.5	20	-	-	43.2	20.4	-	[74]
7	Acetate buffer[b]/ SDS	-	2.2 days	5.5	20	-	-	69	14.4	-	[74]
8	H_2SO_4	5% m	27	-	25	-	-	68	32	60	[76]
9	H_2SO_4	-	2	3.2	60	72	14[d]/4[e]	82			[77]
10	Ac. Ac.[c]	-	2	3.2	60	52	13[d]/9[e]	78			[77]
11	CO_2	4 MPa	0.5	-	120	49	20[d]/4[e]	76		-	[77]
12	CO_2	7 MPa	0.5	3.24	120	53	20[d]/3[e]	77		-	[77]
13	CO_2	8 MPa	0.5	3.2	120	43	20[d]/4[e]	76		-	[77]
14	CO_2	7.5 MPa	2	3.22	100	97	12[d]/4[e]	85		-	[77]
15	ZSM-5	0.01g	2	-	100	48	21[d]/6[e]	73		-	[77]
16	ZSM-5 / CO_2	0.01g 1 Mpa	2	-	100	78	21[d]/4[e]	75		-	[77]
17	-	-	0.5	6.8	120	8	27[d]/15[e]	58		-	[77]

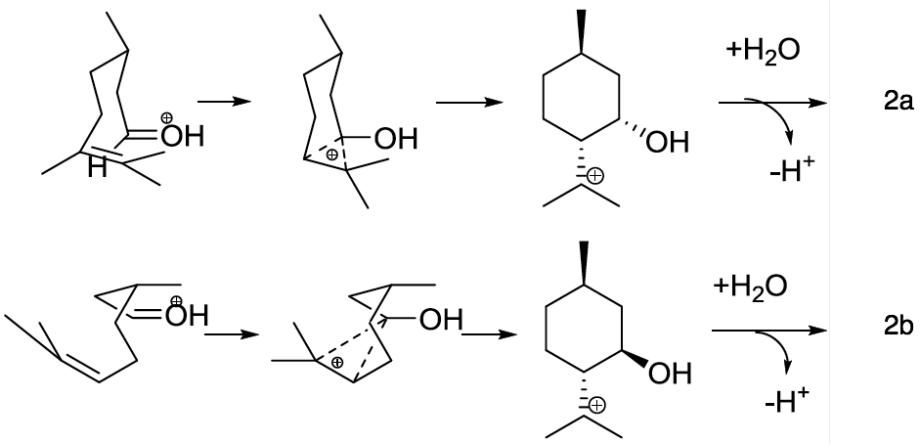
a: low boiling point (citronellal and isopulegol). b: pH 5.5. c: Acetic acid. d: % Isopulegol. e: % Acetals. f: Isolated product

Table 1.2: Summary of main synthesis methods of *para*-Menthane-3,8-diol from citronellal.



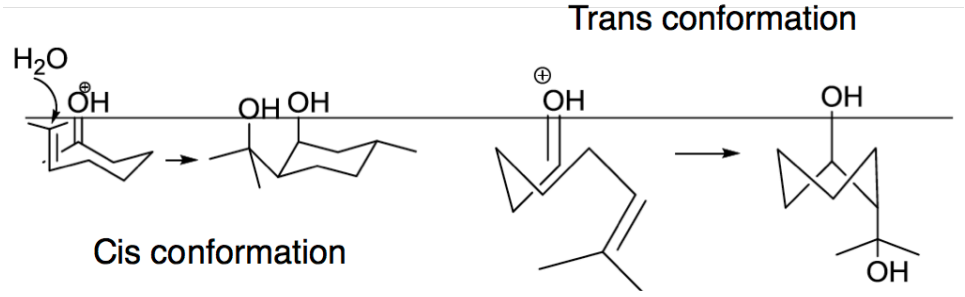
Scheme 1.3: Acetals 3a and 3b formed during the synthesis of *para*-Menthane-3,8-diol with citronellal.

The stereoselectivity in favor of **2a** was explained by Clark Jr. *et al.*[74]. The stabilization of the intermediate carbocation is due to the interaction with the oxygen of the alcohol as shown in Scheme 1.4.



Scheme 1.4: Conformation of the intermediate carbocation during the synthesis of PMD in H_2SO_4 medium.

In an acetate buffer at pH 5.5, the conversion reaches only 64% at room temperature (see Table 1.2, Entry 6). However, under the same conditions and with the presence of a surfactant (sodium dodecyl sulfate, SDS), the conversion reaches 83% (Table 1.2, Entry 7). Since citronellal has a low solubility in water, the surfactant allows the formation of an emulsion, which increases the surface area with the acidic medium (Table 1.2, Entry 7). Clark JR. *and al.*[74] suggest that the carbocation is close to the interface in a "cis" conformation and therefore easier to access for hydration as shown in Scheme 1.5.



Scheme 1.5: Conformation of the intermediate carbocation during the synthesis of *para*-Menthane-3,8-diol in an acidic medium with the presence of a surfactant.

Cheng *et al.*[77] used another method to acidify the reaction medium by working under CO_2 (see Table 1.2, Entries 12-14). Under high pressure (up to 12 MPa), the CO_2 solubilization increases the acidity of the medium. The pH varies as a function of the pressure: 6.8 without CO_2 and around 3.2 at 7-8 MPa. The saturation limit is therefore reached for these pressures. The benefit of this method was studied by the authors with the results obtained with a CO_2 pressure of 7.5 MPa at 100°C (see Table 1.2, Entry 14) and compared to a reaction carried out at the same pH (3.2) with sulfuric acid (see Table 1.2, Entry 9) or acetic acid (see Table 1.2, Entry 10) at 60°C. This comparison shows that the use of CO_2 under pressure at 100°C leads to similar or even better performances for the same reaction times.

1.5. Scope of this Thesis

As explained previously, *para*-Menthane-3,8-diol (PMD) has interesting advantages such as a high repellent activity against mosquitoes, a potential natural origin and it becomes more popular in repellent products the past few years. The aim of this project is to investigate and to improve repellent products based on *para*-Menthane-3,8-diol.

A first part of this thesis focused on the study of PMD, from its synthesis to its introduction into new formulation systems allowing to improve the repellent action against mosquitoes. A green and simple method to produce PMD from the essential oil of *Eucalyptus citriodora* is reported in **Chapter 2**. The repellent activity of the synthesized active is investigated against *Aedes aegypti* mosquitoes and shows a high

repellent action on Human volunteers. The majority of repellent products on the market is composed of mixed surfactant systems or hydro-alcoholic bases, which present several drawbacks for the consumers (skin irritation, reducing time of protection, unpleasant odor or feeling). In **Chapter 3** surfactantless and classical microemulsions containing PMD were studied in order to extend the lasting protection against mosquitoes. A second study in **Chapter 4** involved the development of an oil in water PMD repellent lotion with environmentally acceptable additives. The main goal was to reach a high protection activity comparable to the reference product on the market, the Autan® Protection Plus from SC Johnson composed of KBR 3023. Finally, the complexation of PMD into cyclodextrins (CDs) was investigated in **Chapter 5** in order to solubilize PMD in water and enable its controlled release over time.

In a second part, a new water-soluble repellent active derived from PMD was considered. The low water solubility of PMD restrains the development of simple repellent formulations and the synthesis of the active so called PMD-Succinate (PMD-S) is reported in **Chapter 6**. In order to determine the nature of the new active, especially its surface activity as a surfactant or a hydrotrope, physical and chemical properties were studied in **Chapter 7**. This study is completed in **Chapter 8** by the evaluation of the cytotoxicity of PMD-S on HeLa and Keratinocyte cells as well as those of all the commonly repellent actives used in the market. Finally, an improved synthesis method of PMD-S was investigated with green considerations and an acceptable yield in **Chapter 9**.

In a last part (**Chapter 10**) and independently of the work on PMD and its derivative, a study of 19 Corsican (French island) essential oils was carried out in order to determine their protection activity against mosquitoes as well as their possible integration into repellent products.

Part I

STUDIES OF *PARA*-MENTHANE-3,8-DIOL FROM A NEW SYNTHESIS PROCESS TO ITS INTEGRATION INTO DIFFERENT FORMULATION SYSTEMS

Chapter 2

GREEN SYNTHESIS AND MOSQUITO REPELLENT TESTS OF *PARA*-MENTHANE-3,8-DIOL FROM *EUCALYPTUS CITRIODORA*

2.1. Abstract

A simple and efficient method was developed for the synthesis of *para*-Menthane-3,8-diol (PMD), a well-known repellent active against mosquitoes, from *Eucalyptus citriodora* essential oil and treating it with citric acid in a biphasic medium (H2O/essential oil). As main component, the *Eucalyptus citriodora* contains (+)-citronellal (74% in the present case), which cyclises (Prins reaction) to form cis/trans PMD isomers. As an example, an emulsion containing an aqueous solution of 7% citric acid and *Eucalyptus citriodora* oil at 50°, conducted after 15 hours stirring to 82% conversion of (+)-citronellal with a selectivity of 80% with the remaining presence of monoterpenes and sesquiterpenes in the medium. Investigations of lasting protection on human volunteers were carried out using a cage test bioassay protocol and *Aedes aegypti* mosquitoes. At 20% in iPrOH, the new reaction mixture showed a complete protection of 303 min compared to 22 minutes with the pure essential oil. The modified oil was compared with *N,N*-diethyl-*m*-methylbenzamide (DEET), the most popular active used in repellent formulations. Thermogravimetric analyses (TGA) of *Eucalyptus citriodora*, the modified oil, PMD, DEET, and (+)-citronellal were performed and showed a slow evaporation rate for PMD and DEET as well as for the modified oil, which may explain their long-lasting protection action.

2.2. Introduction

350-500 million cases of malaria, which is transmitted by *Anopheline* mosquitoes, are reported annually and over one million people die, most of them young children in sub-Saharan Africa[78,79]. Prevention and control rely on reducing the number of infected people. A personal protection using repellent products is necessary to minimize the risk of infection and reduce the discomfort caused by mosquitoes. Repellents often contain DEET (*N,N*-diethyl-*m*-methylbenzamide), IR3535 (ethyl 3-[(acetyl)(butyl)amino]propanoate), Icaridin (RS)-sec-butyl-(RS)-2-(2hydroxyethyl)piperidine-1-carboxylate, PMD (para-Menthane-3,8-diol) as synthetics actives[80-82]. However, these synthetic active molecules are often prone to controversial matters due to their potential toxicity. Despite its great protection efficacy, DEET for instance, has been implicated in severe neurotoxicity factors in Human[83-85]. Qui et al.[86] concluded that DEET exhibits a good margin of safety but does manifest some adverse effects. The use of natural repellents is more and more supported by Federal governments or local authorities and demonstrated over the years their potential to replace DEET or others synthetics active, which are active against some mosquito species[87-90]. The use of essential oils extracted from the seeds, the leaves, the branches, the resin of trees or plants as active in repellent formulation is a main interest especially for local market target. Eucalyptus citriodora essential oil is for instance predominately produced in subtropical or tropical countries where, mainly diseases transmitted by mosquitoes (malaria, dengue…) occur[64].

Essential oils possess complex compositions with unique biological activities and have demonstrated a good efficacy against mosquito in the past[91]. Nevertheless, they often showed a short lasting protection and are usually excluded from repellent products as main active[69]. To extend their efficacy and to guaranty the highest protection, two ways are considered. On the one hand, combinations of different essentials oils are employed and then integrated in a proper formulation to prolong the repellent action over time. On the second hand, the pure essential oil might be used as a starting material in a synthesis process to produce new actives with a higher and longer protection efficacy[92].
In the present case, *para*-Menthane-3,8-diol (PMD), a well-known active to repel mosquitoes[93], was synthesized from *Eucalyptus citriodora* oil. It has been found in the

past that PMD is easily obtained from (+)-citronellal (1), which is the main component (74%) in the *Eucalyptus. citriodora* oil[76]. However, these methods are non-reproducible for an industrial process. The cis and trans para-Menthane-3,8-diols isomers (2a-2b-2c-2d) are produced through an acid-catalyzed cyclisation (Prins reaction) of (+)-citronellal as shown on Scheme 2.1, by a treatment of a citric acid aqueous solution[94].
Also, the use of citric acid during the synthesis leads to the production of a natural PMD as long as citric acid is made from a complete biological and natural process. Investigations on different parameters (acid concentration, reaction time and temperature, quantity of H2O in the medium) were performed to reach the best conversion of (±)-citronellal and the highest selectivity of PMD during the reaction. Gas chromatography analyses were performed to identify and quantify each chemical in the new mixture medium.

The modified oil was then subjected to a repellent test on human volunteers by using on a bioassay *Aedes aegypti* mosquitoes. Investigations involved the use of DEET as well as pure PMD and pure *Eucalyptus citriodora* essential oil as a test comparison with the new reaction mixture.

Finally, thermogravimetric analyses (TGA) were performed on *Eucalyptus citriodora* as well as on *para*-Menthane-3,8-diol, *N,N*-diethyl-*m*-methylbenzamide and on the new mixture reaction to determine the weight loss of selected samples at 33° (skin temperature) versus time. These records provided information on the evaporation rate of these different samples, which were compared with their repellent action on mosquitoes.

2.3. Experimental procedures

2.3.1. Synthesis and Analysis

Chemicals *Eucalyptus Citriodora* essential oil (Albert Vieille, France) was extracted by hydro distillation in China. Table 2.1 sums up the list of different compounds present in the oil (data given from the manufacturer). 2-hydroxypropane-1,2,3-tricarboxylic acid monohydrate (citric acid; Sigma Aldrich, Germany; grade: 99.5%), was used as a catalyst

for the Prins reaction and dissolved in distilled H_2O to obtain solutions at different concentrations. Sodium sulphate (Na_2SO_4; Merck, Germany; grade: 99%) was used as a drying agent, *N,N*-dimethylmethanamide (DMF; Merck, Germany; grade: 99.8%) as a solvent to analyse the mixture reaction by gas chromatography. Isopropyl alcohol (^{i}PrOH; Merck, Germany) was used as a solvent for the modified oil, the pure oil and the PMD to test their repellent action in cage tests. 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Taksago, France) was used with a specific ratio between the (+)-cis and (-)-trans isomers (62/38) according to the manufacturer. At last, *N,N*-diethyl-*m*-methylbenzamide (DEET: Sigma Aldrich, Germany) was used as received.

Component	% w/w	Component	% w/w
α-Thujene	0.04	Linalool	0.22
α-Pinene	2.10	Neoisopulegol	2.25
β-Pinene	1.28	Isopulegol	4.68
Sabinene	0.07	*β*-Caryophyllene	1.13
Myrcene	0.14	Terpinene-1-ol-4	0.16
α-Terpinene	0.08	*α*-Terpineol	0.09
Limonene	0.17	Citronellol	5.95
Cineol-1,8	0.49	Citronellyl acetate	1.33
Cis-*β*-Ocimene	0.09	Nerol	0.06
γ-Terpinene	0.16	Geranial	0.03
ρ-Cymene	0.07	Geraniol	0.1
Terpinolene	0.16		
Citronellal	74.21	**Sum of component**	**95.06**

Table 2.1: Chemical composition of *Eucalyptus Citriodora* essential oil

Synthesis of *para*-Menthane-3,8-diol isomers In a 50-ml round-bottomed flask 5.25-22 g of a 1-15 wt% citric acid aqueous solution and 3.7 g (24.0 mmol) of *Eucalyptus citriodora* oil were charged and heated between 40 to 60°C under agitation (450 tr/mn). The biphasic medium was maintained at constant temperature between 6 to 15 hours. Then, the organic phase, composed of PMD, monoterpenes, sesquiterpenes and others acetals, were separated from the aqueous phase after several hours decantation and dried with sodium sulphate (Na_2SO_4).

GC Analysis GC analyse of the mixture medium were performed on a HP 6890 Series gas chromatograph fitted with a flame ionization detector (FID) and an electronic integrator. The capillary column used was a HP model 19091 J-413: HP-5 5% Phenyl Methyl Siloxane (30 m x 320 μm; film thickness 0.25 μm nominal). Helium was employed as a carrier gas at a flow rate of 104 ml/min and 65.6 kPa inlet pressure. Temperature program was: 50°C at 10°C/min, rising to 225°, and then remaining at 225°C for a period of 15 min. Injector and detector were maintained respectively at 280°C and 250°C. Samples (1 μL) were injected neat with a 50:1 split ratio.

Identification and quantification method All components were identified by the retention time of standard molecules from a specific GC program. The response factor of the internal standard solution was measured using GC with known amount of *N,N*-dimethylmethanamide and of reaction mixture.

Thermogravimetric analysis Pure *Eucalyptus citriodora* oil, *para*-Menthane-3,8-diol and the new reaction mixture samples of ≈13 mg were subjected to thermogravimetric analysis in a nitrogen atmosphere. A TGA7 from Perkin Elmer corp. (USA-Norwalk) was used. An isotherm heating program was settled up at 33°C (Human skin temperature on the forearm). In order to obtain a low-noise TG signal, a constant gas flow of 70 ml min^{-1} was set for all the tests. The precision of temperature measurement for the thermobalance is ± 1°C. The continuous records of weight loss and temperature were obtained and used to determine the evaporation rates (weight-loss % min^{-1}) of the pure essential oil, PMD, DEET, (+)-citronellal and of the modified essential oil.

2.3.2. Mosquito repellent studies

Insects Strain of *Aedes aegypti* mosquitoes from Bayer AG were reared according to the standard protocol at 27°C, a relative humidity of 60–80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in H2O filled with a 1:1 mixture of tap- and deionised H_2O, and fed with fishfood flakes (Tetra Min®). Before hatching the pupae were transferred to a cage (40 x 30 x 20 cm) and provided with sugar soln. (10% dextrose). Mosquitoes at an age of 9-14 days were used for the tests.

Volunteers Five human volunteers aged between 20 and 26 participated in the mosquito cage test bioassay. No abnormal allergic reaction after application of the formulations was observed.

Laboratory tests Human skin tests were conducted as described below.

Application of repellents: The skin of the forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% iPrOH and then dried with a paper towel. An area larger than the test window was marked on the skin with a metal template. According to the United States EPA, 1 g of pump spray is applied per 600 cm² skin. The marked area had a size of 98 cm²; therefore 0.2 g were applied to the test area. The test substance was applied using a pipette with disposable tips, for each test person a new tip was used. The test substance was spread evenly on the skin of the forearm wearing a latex glove.

Exposure to mosquitoes: Thirty mosquitoes were placed in a test cage that was fitted with a test window in the floor of the cage. The test window was closed by a metal slide and was opened by inserting a metal frame for the exposure of the treated quadratic (98cm²) skin area (ventral) and untreated area (back) of the volunteer´s forearms. This method was designed so that each volunteer served as his own control.

Zero control: Zero control is the untreated back of the forearm of the test person. A window frame with mosquito net is used to keep probing mosquito from successfully taking a blood meal. Biting pressure must exceed 10 probings in 30 s. After 30 s and more than 10 probings the mosquitoes were considered as active and suitable for the experiment.

Test proper: Each test person was assigned to one test cage. Between two tests a special air ventilation system was attached to the cage in order to prevent any accumulation of odours and active substances in the cage. The treated skin was exposed to the mosquitoes for a testing time of 2 min. In this time, the number of landings and bitings on the treated skin was noted and compared to the untreated control skin.

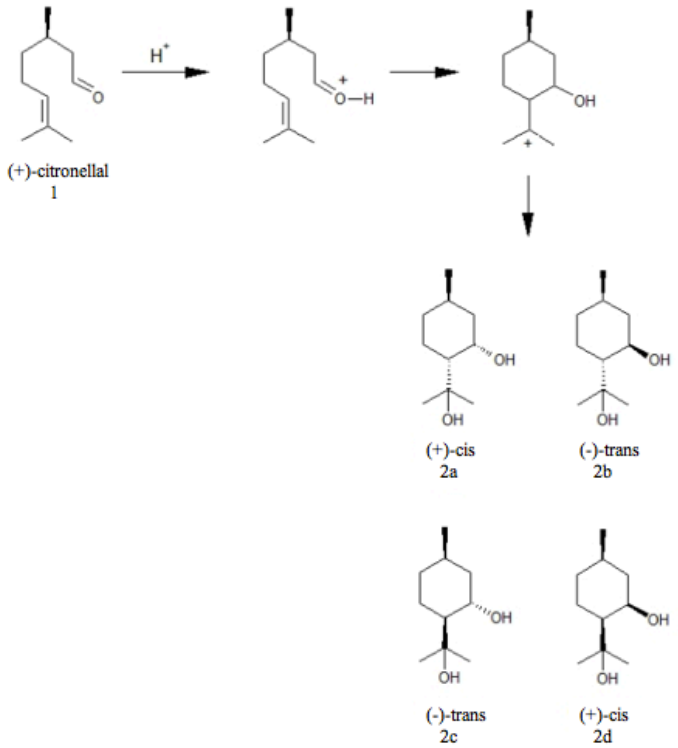
Determination of duration of protection: As far as there is no official protocol in EU, tests were based on the EPA draft guideline OPPTS 810.3700 (EPA, 2000). The criteria to define a complete protection time were dependent on the zero control. A 15 minutes margin of error was attributed on every result.

Statistical analysis Data collected during evaluation of duration-of-protection tests were subjected to an analysis of variance (ANOVA) and *t*-test ($p < 0.05$) using the software SPPS (version 12.0 for windows). This statistical analysis was used to check the reliability of the results obtained from the bioassay and to verify if there are any significant differences in the documented protection times of the tested solutions.

2.4. Results and discussion

2.4.1. Transformation of Eucalyptus citriodora oil and synthesis of para-Menthane-3,8-diol isomers

The preparation of *para*-Menthane-3,8-diol isomers from (+)-citronellal contained in the *Eucalyptus citriodora* oil involves the presence of an acidic medium as shown on Scheme 2.1. Citric acid was considered due to its natural production source[95] for the Prins reaction as a catalyst for the cyclisation of (+)-citronellal, which lead to a more natural way for the PMD synthesis.



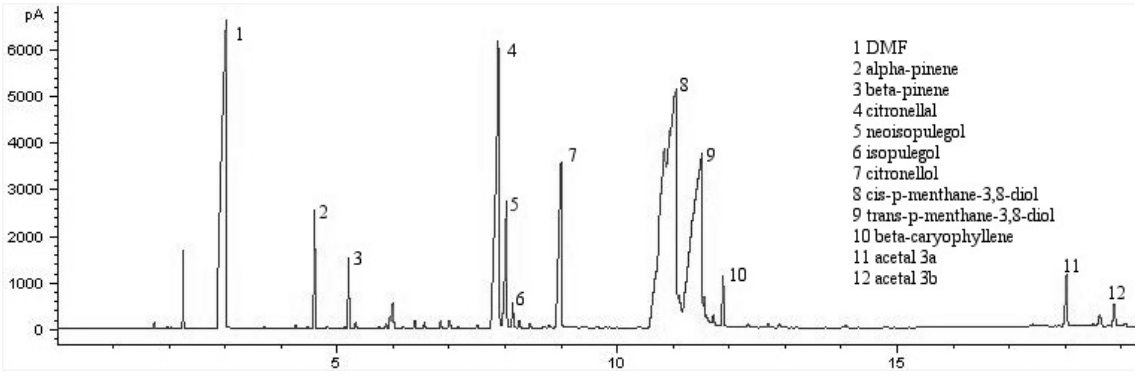
Scheme 2.1: Acid-catalyzed cyclisation of (+)-citronellal. Synthesis of *cis* and *trans*-(±)-*para*-Menthane-3,8-diol.

H_2SO_4 was already used in the past to produce *para*-Menthane-3,8-diol from pure (+)-citronellal[70]. The present work shows the production of the same active through a similar reaction but using as a starting material, *Eucalyptus citriodora*. This oil contains more than 24 compounds, mainly monoterpenes and sesquiterpenes, and the majority may react in such acidic medium and might involve the apparition of others entities (side products). In this study, different parameters were investigated during the cyclisation reaction: the acid concentration, the temperature of the medium, the reaction time and the quantity of the aqueous solution. All these parameters were optimized and contributed to improve a mean of the conversion of citronellal and then reached the highest selectivity of PMD during the reaction. The results are shown in Table 2.2.

Run	Concentration Acid (%)	Temperature (°C)	Time (hours)	Water weight (g)	Conversion of (+)-citronellal (%)	Selectivity of PMD (%)	Ratio (-)-cis / (-)-trans
1	1	50	15	5.25	46.3	90.3	68 / 32
2	5	50	15	5.25	81.2	66.7	71 / 29
3	7	50	15	5.25	82.1	79.6	65 / 35
4	10	50	15	5.25	83.7	78.9	68 /32
5	15	50	15	5.25	77.7	76.9	66 / 34
6	7	50	6	5.25	55.1	85.7	68 /32
7	7	50	9	5.25	67.2	82.8	66 / 34
8	7	50	15	10.5	85.3	78.2	66 /34
9	7	50	15	17.5	82.8	79.2	65 / 35
10	7	50	15	22	84.8	80.4	66 /34

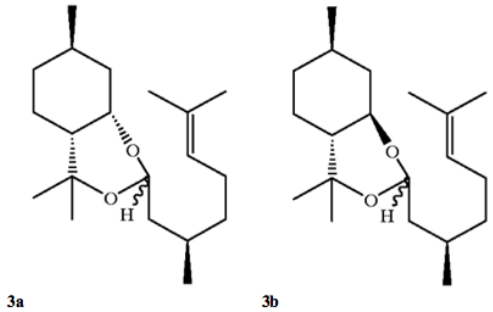
Table 2.2: Concentration of citric acid, temperature, reaction time and quantity of water in the medium, are parameters considered for the cyclisation of (+)-citronellal. Conversion and selectivity were calculated from appropriate programs.

Table 2.2 shows clearly the conversion of (+)-citronellal as function of different parameters. Run n°3 led to the best production of *para*-Menthane-3,8-diols with 82% conversion of citronellal and 80% selectivity of PMD. Run n°9 provided similar results but it needed 3 times more acidic medium to reach the same selectivity. It was necessary to stir in a 50° bath for 15 hours, which is conceivable for a mass production perspective. The synthesis is perfectly reproducible even if the stirring action is highly determinant to provide the best conversion. The modified oil was subjected to GC analysis using a method of choice and then calculations were done to determine the composition of the reaction mixture. An example is shown in Graph. 2.1 with the production of *para*-Menthane-3,8-diol using a 7% of citric acid solution for 15 hours at 50° (run n°3).



Graph. 2.1: GC chromatogram of the synthesis of *para*-Menthane-3,8-diol from *Eucalyptus citriodora* oil. Reaction using 7% of citric acid at 50° for 15 hours (run n°3). GC parameters are described in the Experimental section.

Peaks 8 and 9 correspond respectively to the (+)-cis and (-)-trans *para*-Menthane-3,8-diols. (-)-cis and (+)-trans are also present in the reaction mixture at a very low concentration. Around 13% of (+)-citronellal remained in the solution, which match a conversion of 82%. The selectivity for the PMD production reached 80% with 64% concentration in the final mixture ((+)-cis/(-)-trans: 65:35) on run n°3. Citronellol (peak 7) remained in the modified oil at 5.95% as well as iso/neosipulegol (peaks 5/6) with respectively 4.7% and 2.2% of the total mixture. Additionally, PMD acetals 3a and 3b (see Scheme 2.2) were produced at a very low concentration, respectively 1.40% and 0.35%, as already found in the literature[75]. Several syntheses were done using others parameters showing a large concentration of these acetals at the end, which is not the case on run 3. There is no evidence that these acetals may provide any repellent action on mosquitoes.

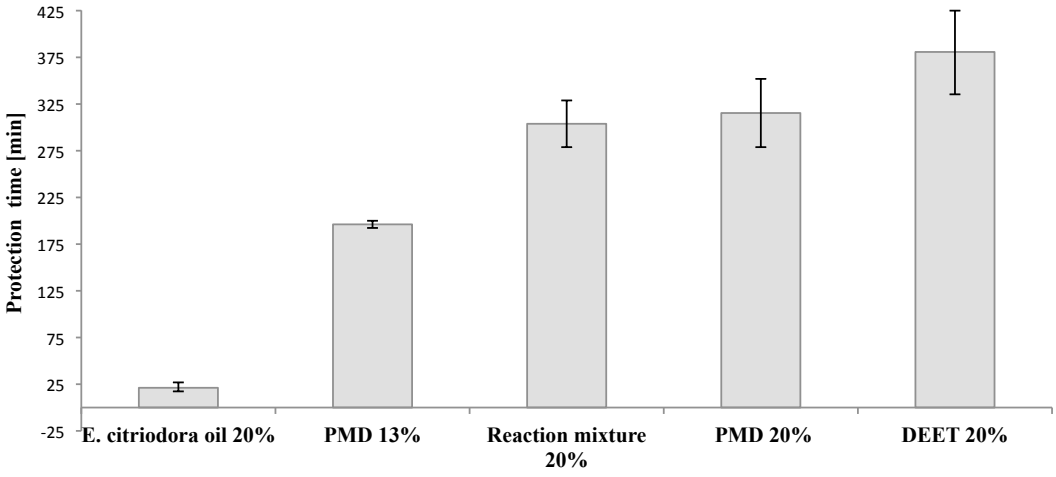


Scheme 2.2: Structure of two PMD acetals produced during the cyclisation of (+)-citronellal (peaks 11/12 on Graph. 2.1).

As shown on Graph. 2.1, many monoterpenes and sesquiterpenes remained in the modified oil. The authors assumed that their concentrations stayed constant in such acidic medium compared to the original oil. The modified oil was not subjected to any separation process and was then tested with this composition on a bioassay to assess its repellent action on *Aedes aegypti* mosquitoes.

2.4.2. Mosquito repellent study

This study evaluated the repellence of the new reaction mixture by using cage tests on a bioassay with *Aedes aegypti* mosquitoes. The screening was set up following the method previously described (Experimental part). On two days test, Human volunteers applied five solutions on their forearms: a 20% of pure *Eucalyptus citriodora* oil, 13% and 20% of pure *para*-Menthane-3,8-diol as both standard control, a 20% DEET and a 20% solution of the new modified oil. The new mixture was obtained from the process previously described as n°3 in Table 2.2. Graph. 2.2 exhibits the results of the different samples used in the cages tests showing great differences on the time protection.



Graph. 2.2: Different protection time values on human volunteers with *Aedes aegypti* of solutions previously described.

As a result, the pure essential oil solution has shown a short repellent activity on *Aedes aegypti* with a mean protection time of 22 minutes, which is less than previous figures

found in the literature. *E. citriodora* has been credited with anti-inflammatory, antibacterial, and antifungal activity, and, recently has gained popularity as an insect repellent[96]. In the present study, this oil has never proved a great repellent action at any specific concentration (unpublished data) in contrast to all the data found in the literature. It is mainly employed in commercial products as a secondary active in addition to others actives. Besides, thermogravimetric analysis will show further in this paper its high evaporation rate, which may affect its lasting protection.

196 minutes was reached with the pure *para*-Menthane-3,8-diol solution at 13% concentration and 315 minutes for the 20 %, which corresponds to the mean values of PMD protection time at these concentrations as shown on previous reports[57]. PMD has a faint mint odour and is slightly soluble in water.

DEET was also tested on a bioassay since this component is used in many commercial products all over the world. The 20% concentrated solution provided 380 minutes complete protection against *Aedes aegypti* mosquitoes using the same mosquito strain and protocol. In the literature, PMD preparations appear to provide a lower protection time than DEET (up to 1.5 lower) and require more frequent reapplication to maintain the same level of protection[65].
In comparison to pure *Eucalyptus citriodora* oil, the modified oil caused a great increase on the time protection on human volunteers against *Aedes aegypti* with a mean value of 303 minutes.

ANOVAs conducted on the protection time of these 5 samples revealed no significant differences between the modified oil, the DEET and PMD solutions concentrated at 20%. On the contrary, statistics calculations showed a significant difference between the new reaction mixture (PMD 13% + mono/sesquiterpenes + acetals) and the 13% pure PMD solution from Takasago with an improvement up to 50% on the protection time.

The modified oil is mainly composed of PMD cis/trans isomers (64%), (+)-citronellal (13%), citronellol (6%) and many others terpenes in lower concentrations. However, the presence of these side products and residues do not affect the repellency of PMD and even increases drastically its action. Unpublished data show that individually, these molecules in alcoholic solutions and using the same protocol, present no efficient

protection over time against *Aedes aegypti* mosquito at such low concentration. Besides, investigations were carried out with combinations of PMD/citronellal and PMD/citronellol with the same concentrations as they are present in the modified oil. The results indicate no significant differences between these two combinations and pure PMD solutions.

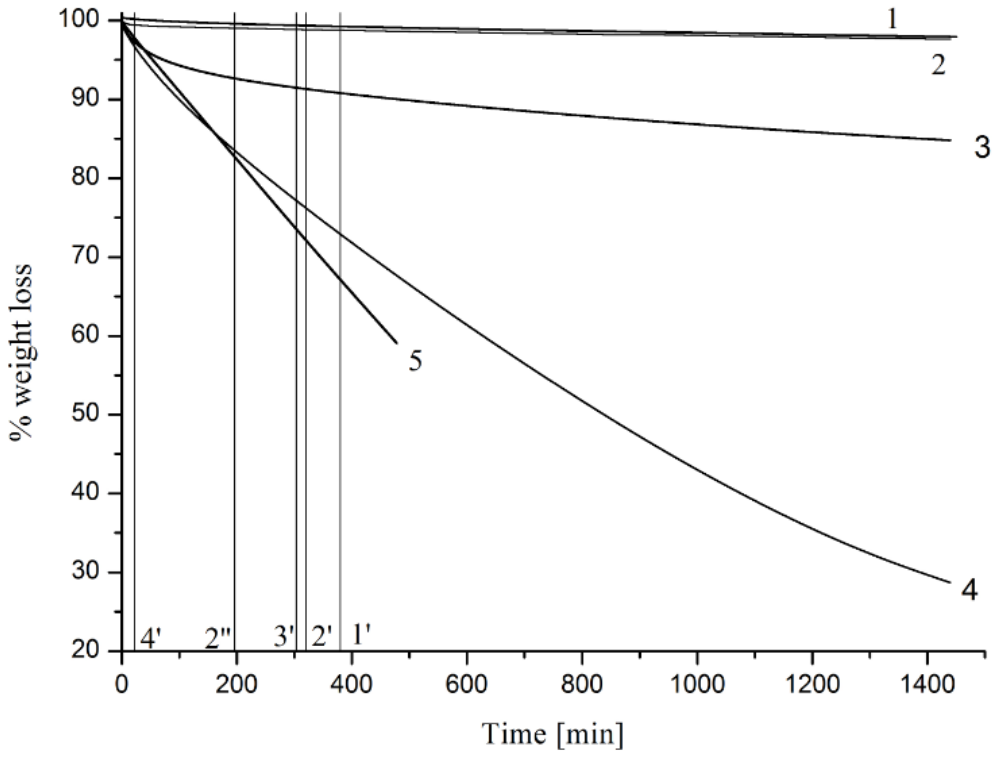
There is no obvious explanation on the high protection action of the modified oil compared to the pure PMD except that the side components may act in synergism all together to provide a certain protection even if they did not show any repellent properties separately.

2.4.3. Thermogravimetric analysis

TGA thermograms for DEET, PMD, *Eucalyptus citriodora oil*, *Eucalyptus. citriodora* modified and (+)-citronellal are given in Graph. 2.3. From the thermogram, it is found that DEET and PMD undergo the same loss of weight over time at 33 °C to reach about 2% loss after 24 hours. This behavior is directly related to the vapor pressure of the two molecules, which is comparable. PMD and DEET have both a low vapour pressure inducing a weak volatility. (0.00109/ 0.0056 mm Hg at 25°C) [vapor pressure calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (Ó 1994-2008 ACD/Labs). Despite their similar volatility over time, these two molecules display a different behaviour on the mosquitoes as shown on Graph. 2.3. This difference is not significantly different. The lost of activity is certainly due to the penetration of these two components through the skin. The PMD molecule is much smaller than the DEET and may cross easily the lipid layers of the skin. On the other hand, the hydroxide groups of PMD might interact with the polar heads of the lipids and involve a highest retention of the PMD molecules on the upper skin layers as for the DEET. Also, the receptors' sensitivity to the mosquito's antennas might be a parameter to explain the difference of behaviour between these two molecules.

TGA of the *Eucalpytus citriodora* essential oil exhibits a completely different curve. After 24 hours, 70% of the oil evaporated. Essential oils usually have relatively high vapor pressures inducing high volatilities over time. Hydrocarbonated monoterpenes are

very well known to display a high evaporation. The 6% terpenes present in the *Eucalyptus citriodora* oil may evaporate first and should be responsible for the weak protection against the mosquitoes (around 25 minutes). The citronellal curve shows a linear degradation over time, which is significant over a long period. A repellent study (not presented) on this molecule did not show any repellent action on *Aedes aegypti* mosquito, in contrast to others studies found in the literature.



Graph. 2.3: TGA curves of the evaporation rate of DEET (1), PMD (2), modified *Eucalyptus citriodora* oil (3), *Eucalyptus citriodora* oil (4) and pure (+)-citronellal (5) under a nitrogen atmosphere at 33°C ± 1 °C. The vertical lines represent the protection time obtained from the repellent study for the DEET solution (1'), the PMD solution at 20 wt% and 13 wt% (2'-2''), the modified oil (3') and the pure *Eucalyptus citriodora* oil (4').

Finally, the modified oil was subjected to a TGA analysis. After 24 hours at 33°, the reaction mixture lost 15% of its weight showing a very slow degradation. This oil is composed of around 64% PMD, 13% citronellal and the rest of hydrocarbonated monoterpenes or sesquiterpenes and acetals. The first losses are induced by the terpenes as previously mentioned. The thermogram presents a plateau reached after few hours as also found with the PMD and DEET analysis. The presence of PMD in the reaction

mixture and its low degradation logically displays a good protection time on the mosquitoes at around 300 minutes for a 20% in an alcoholic solution. However, the presence of 13% of citronellal in the solution should lead to a greater deformation of the curve, which is not the case. The authors recall that PMD may self-associate in hydro-alcoholic solutions and form aggregates, which could slow down the skin rate penetration and then provide a longer repellency action.

2.5. Conclusion

para-Menthane-3,8-diols were synthesized from *Eucalyptus citriodora* oil by using an easy, fast, green and cost-effective process. With adapted choices of acid, temperature and time reaction, cyclisation of (+)-citronellal from the oil induces the production of cis and trans-*para*-Menthane-3,8-diols with a high selectivity. In a bioassay, this natural PMD in combination with others acetals and terpenes gave a long-lasting protection against *Aedes aegypti* mosquitoes. 303 minutes of complete protection on human beings was reached with the new mixture (20% in ^{i}PrOH). A comparison with the reference active DEET and the modified oil showed no significant difference in the protection time between both actives at the same concentration.

Chapter 3

EFFECTIVE INSECT REPELLENT FORMULATION IN BOTH SURFACTANTLESS AND CLASSICAL MICROEMULSIONS WITH A LONG-LASTING PROTECTION FOR HUMAN BEINGS

3.1 Abstract

The purpose of this work is to develop a new generation of repellent products with a long-lasting protection based on a natural component, *para*-Menthane-3,8-diol (PMD). The active is first rendered soluble in a surfactantless microemulsion (water/isopropyl alcohol/PMD) and then in classical microemulsions. The presence of self-associated nanostructures is detected by Dynamic Light Scattering. A synergetic system of surfactants (Cremophor® RH40 and Texapon® N70) is used. Additionally, 2-ethylhexanediol-1,3 and ethyl (S)-(-) lactate are incorporated. The final product contains as main components, 46% of water, 25% of isopropanol, 20% of non-water soluble PMD and only 4% of surfactants. Investigations of lasting protection on human volunteers are carried out using a cage test bio-assay protocol and *Aedes aegypti* mosquitoes. A complete protection of 315 minutes is found on the test persons using the surfactantless microemulsion. An extension is observed with the final formulation to reach a mean of complete protection of 385 minutes. This study demonstrates that alternative formulations using a natural active instead of synthetic chemicals like *N,N*-diethyl-*m*-methylbenzamide (DEET) can be efficient for human protection against mosquitoes.

3.1. Introduction

Mosquitoes cause more human suffering than any other organism by carrying a large variety of diseases including malaria, dengue, yellow fever, chikungunya etc. and infect millions of people each year[78]. More than 500 million Malaria cases occur every year, which cause the death of 1 million people, including 1 child every 30 seconds[79]. To anticipate any kind of epidemic diseases or to avoid any discomfort provided by the mosquitoes, a personal protection using repellent products is necessary to minimize the risk of an infection and to reduce the discomfort caused by mosquitoes.

Commercial insect repellents containing the active ingredient *N,N*-diethyl-*m*-methylbenzamide (DEET) are very common on the market, because of their long-lasting protection of human being[97,98]. Recently, DEET has been implicated as a factor in neurotoxicity illness due to a large absorption of the chemical through the skin[83]. Despite its excellent repellency, consumers are concerned about DEET and its toxicity and may use alternative chemicals with a lower activity but safer properties[62,99]. Research has been carried out for decades to find other chemicals and several tracks were led on natural components. Numerous essential oils were found to possess repelling properties, though the efficacy and protection levels are varying[88]. Only five components were regulated by The Environment Protection Agency (EPA) and approved by The center for Disease Control and Prevention (CDC) as an active ingredient for repellent formulations of skin care products with a "low toxicity" and a "safe protection"[89,90]. Three of them are considered as synthetic actives like DEET, KBR 3023 (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester)[100] and IR3535 (ethyl ester 3-[N-Butyl-N-acetyl]-aminopropionic acid)[101]. Summaries from EPA indicate that KBR 3023 is slightly toxic by eye, dermal and oral routes and IR3535 shows no harmful toxicity when ingested, inhaled, or used on skin. Two others chemicals are regulated as natural active substances: oil of citronella (Java type) containing mainly citronellal and *para*-Menthane-3,8-diol (PMD)[72]. The first one shows little or no toxicity, but may cause skin irritation whereas PMD has no adverse effects except for eye irritation.

In the purpose of formulating a repellent product with a natural active ingredient inside, PMD and Citronella oil were the only two valuable substances, regarding the different

regulation commissions on this specific market. Preliminary tests in a bio-assay have shown a certain repellent efficacy for both chemicals with a great potential for PMD. In comparison to PMD, Citronella oil provides shorter protection time, therefore this work focusses on PMD as an active ingredient for a new repellent formulation. *para*-Menthane-3,8-diol is a natural component extracted from the leaves of *Eucalyptus Citriodora* shrubs. PMD has a faint mint odour, a low volatility (0.00109 mm Hg at 25°C) and is slightly soluble in water [vapour pressure calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris (© 1994-2008 ACD/Labs)]. PMD repellent properties were first established in the 1960's[80]. Previous studies have shown that the isomers (see Fig. 1) display different repellent properties and, in particular, that the (±)-trans isomers initially has the better repellency, whereas the (±)-cis isomers have a longer lasting effect[94].

This work focuses on PMD formulations and their protection efficacy in a cage test bio-assay. The cage test is performed with *Aedes aegypti* mosquitoes on volunteers using different concentrations of PMD in isopropanol. Secondly, PMD is incorporated in several microemulsion formulations. Dynamic Light Scattering (DLS) measurements have shown the possibility to form "surfactantless" and "classic" microemulsions. Two different kinds of microemulsions have been reported in the past. One, so called "classical microemulsion", consists of a homogeneous and clear solution composed of water and one or more lipophilic compounds in combination with one or a mixture of surfactants[102]. Microemulsions have been also found in surfactantless solutions containing only water, alcohol and hydrophobic compounds. According to the literature these microemulsions are called "surfactantless or detergentless microemulsions"[103,104].

Formulations are made to provide a time protection of around 6 hours and to render soluble PMD in presence of relatively high quantity of water. Additives like 2-ethylhexanediol-1,3 and ethyl (S)-(-) lactate were incorporated in the formulation. These two additives were added to reduce the quantity of alcohol in favor of water in the medium and to improve the lasting protection from mosquito bites.

3.2. Experimental procedures

3.2.1. Formulation and characterization

Chemicals 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Taksago, France) was used with a specific ratio between the (+)-cis and (-)-trans isomers (62/38) according to the manufacturer (see Figure 1). Previous studies have demonstrated this ratio to provide the highest repellent properties on two mosquito species *Anopheles stephensi* and *Aedes Aegypti*[94]. PEG-40 hydrogenated Castor oil (Cremophor® RH40; BASF, Germany), Lauryl ether sulfate (Texapon® N70; Cognis, Germany), 2-ethyl-1,3-hexanediol (Merck, Germany; grade: 99.9%). Ethyl (S)-(-) lactate (Sigma Aldrich, Germany; grade: 98%) and isopropyl alcohol absolute (Mallinckrodt Baker, Germany; grade: 99.9%).

Dynamic Light Scattering measurements Particle size were performed using a commercial goniometer (CGS-2, ALV-GmbH Langen, Germany) equipped with a vertical-polarized 22 mW HeNe laser (wavelength = 632.8 nm), a fiber optical detection unit with an avalanche photodiode, and an ALV-5000/E multiple correlator. Cylindrical light-scattering cells of 10 mm outer diameter were used. All measurements were taken at 90.028° detection angle and at 25°C. The solutions prepared for light scattering were filtered using a syringe filter with 200 nm pore size. All solutions were left to equilibrate at least 24h before measurements were taken. An automatic process was performed with a 10 runs method for each sample. The particle sizer and its attendant software (ALV-5000/E-Win V1.4.8.by ALV-GmbH Langen, Germany) provided first the time correlation function of the scattered intensity. The decrease of this correlation function with displacement time (called “lag time”) can be used to extract information about the diffusion coefficient of a particle or droplet in solution. The measured diffusion coefficient can be used to calculate a hydrodynamic radius (Rh) of the droplet using the Stokes–Einstein equation:

$$R\mathrm{h} = \frac{kT}{6\pi\eta D}$$

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the continuous phase and D is the diffusion coefficient. The data are first analyzed by cumulant analysis to obtain an average diffusion coefficient and subsequently by CONTIN analysis in order to obtain information about the entire distribution of the particle size (monomodal or multimodal)[65,69].

3.2.2. Preparation of microemulsions

Surfactantless microemulsion preparation The possible domain of existence of the surfactantless microemulsion was determined by visual observations according to the procedure described by Clausse et al., 1987[105]. This domain matches with the domain of existence of the solution showing a monophasic and clear phase in the water/PMD/isopropanol ternary phase diagram.

Regular microemulsion preparation The surfactants system was first prepared in 10 ml test glass tubes. The active substance (PMD), the solvent (isopropyl alcohol), the additives (2-ethyl-1,3-hexanediol, Ethyl (S)-(-) lactate) and finally the water were added. The microemulsion was blended with a mixer and slightly heated if necessary until a clear solution was obtained.

Microemulsion stability The microemulsion stability was checked by keeping the formulations at room temperature without any stirring action. No destabilization was observed after a period of 8 months.

3.2.3. Mosquito repellent studies

Insects Strain of *Aedes aegypti* mosquitoes from Bayer AG were reared according to the standard protocol at 27°C, a relative humidity of 60–80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in H_2O filled with a 1:1 mixture of tap- and deionised H_2O, and fed with fishfood flakes (Tetra Min®). Before hatching the pupae were transferred to a cage (40 x 30 x 20 cm) and provided with sugar soln. (10% dextrose). Mosquitoes at an age of 9-14 days were used for the tests.

Volunteers Five human volunteers aged between 20 and 26 participated in the mosquito cage test bioassay. No abnormal allergic reaction after application of the formulations was observed.

Laboratory tests Human skin tests were conducted as described below.

Application of repellents: The skin of the forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% iPrOH and then dried with a paper towel. An area larger than the test window was marked on the skin with a metal template. According to the United States EPA, 1 g of pump spray is applied per 600 cm^2 skin. The marked area had a size of 98 cm^2; therefore 0.2 g were applied to the test area. The test substance was applied using a pipette with disposable tips, for each test person a new tip was used. The test substance was spread evenly on the skin of the forearm wearing a latex glove.

Exposure to mosquitoes: Thirty mosquitoes were placed in a test cage that was fitted with a test window in the floor of the cage. The test window was closed by a metal slide and was opened by inserting a metal frame for the exposure of the treated quadratic (98 cm^2) skin area (ventral) and untreated area (back) of the volunteer´s forearms. This method was designed so that each volunteer served as his own control.

Zero control: Zero control is the untreated back of the forearm of the test person. A window frame with mosquito net is used to keep probing mosquito from successfully taking a blood meal. Biting pressure must exceed 10 probings in 30 s. After 30 s and more than 10 probings the mosquitoes were considered as active and suitable for the experiment.

Test proper: Each test person was assigned to one test cage. Between two tests a special air ventilation system was attached to the cage in order to prevent any accumulation of odours and active substances in the cage. The treated skin was exposed to the mosquitoes for a testing time of 2 min. In this time, the number of landings and bitings on the treated skin was noted and compared to the untreated control skin.

Determination of duration of protection: As far as there is no official protocol in EU, tests were based on the EPA draft guideline OPPTS 810.3700 (EPA, 2000). The criteria to define a complete protection time were dependent on the zero control. A 15 minutes margin of error was attributed on every result.

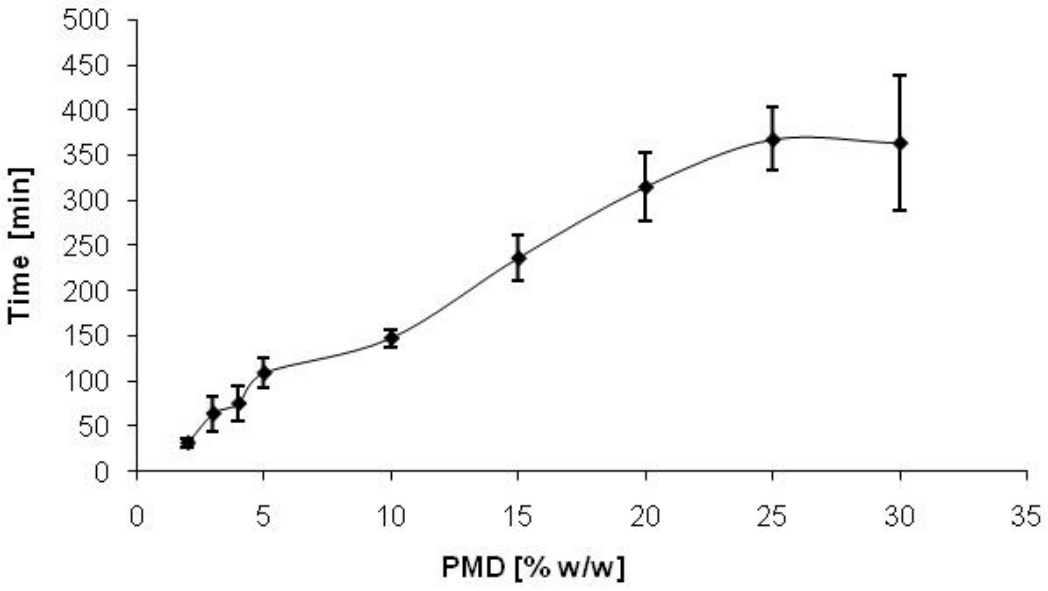
3.2.4. Statistical analysis

Data reported time of protection were subjected to an analysis of variance (ANOVA) and Post-Hoc T-test ($P<0.05$) using the software IBM SPSS Statistics (version 22 for windows). This statistical analysis was used to check the reliability of the results obtained from the assay.

3.3. Results and discussion

3.3.1. Dose effect of PMD in isopropanol on a bio-assay

Several cage tests were performed with concentrations between 2 to 30% w/w of *para*-Menthane-3,8-diol in isopropanol. Graph. 3.1 illustrates the concentration dependant profile for PMD on the protection time with Aedes Aegypti mosquitoes. The protection times prolonged gradually as concentration increased. A plateau seems to be reached at 25 % weight of PMD with a lasting protection of 367 minutes. However, the existence of this plateau remains hypothetic due to a large standard error between each experiment and especially at a 30% w/w PMD.



Graph. 3.1: Time of protection until 3 bites were recorded on volunteers´ skin as a function of PMD (weight %) concentration in isopropanol.

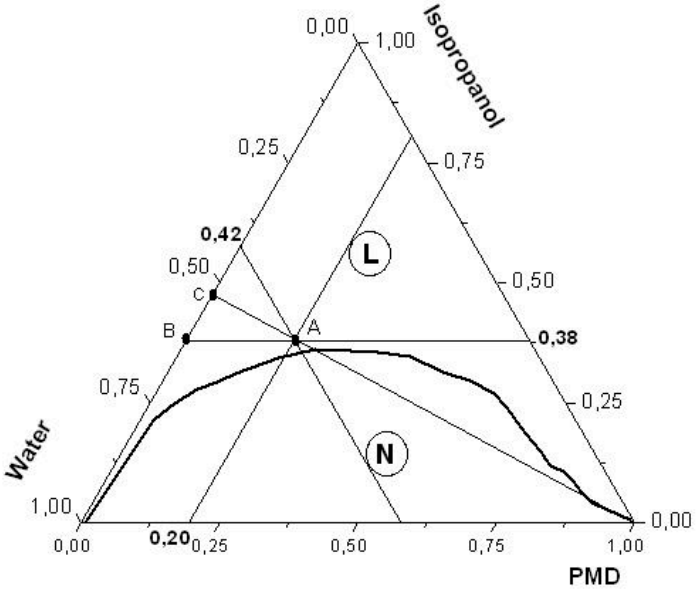
Today, the most common ingredient in commercial insect repellents is *N,N*-diethyl-*m*-methylbenzamide (DEET) and is used by 30% of the population each year[106]. In the past, several studies were carried out to evaluate the level of protection for *para*-Menthane-3,8-diol and DEET in commercial products on the same bio-assay[107]. The experiments showed a better lasting protection for DEET with a factor of 1.5 compared to PMD[108]. The majority of DEET repellent products contain 15% w/w of the active ingredient *N,N*-diethyl-*m*-methylbenzamide. Based on this information, 20% w/w PMD was chosen for the solution as a convenient concentration for its integration in a proper formulation to provide up to 7 hours of complete protection against *Aedes Aegypti* mosquitoes.

3.3.2. Solubility of PMD in a hydro- alcoholic system: "surfactantless microemulsion"

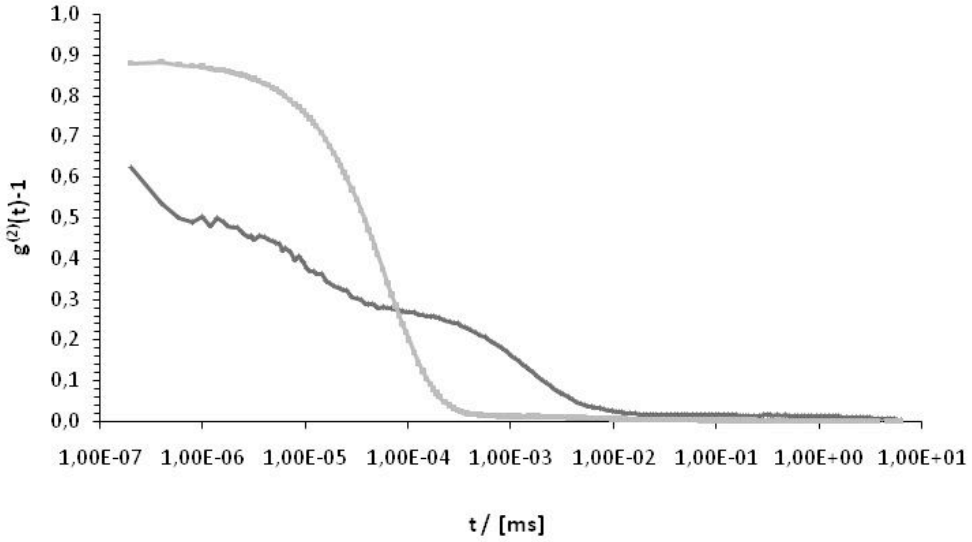
A phase diagram of the three components system, PMD/isopropanol/water was constructed (see Graph. 3.2). A large area (L) of existence of clear and homogeneous solutions is observed. This extended area is due to the fact that water and isopropanol are co-solvents and isopropanol and PMD are also co-solvents. The limiting factor for a greater extend of L is the poor solubility of PMD in water and of water in PMD. The boundary between L and the domain showing two separated phases (area N) looks like a horseshoe. This shape reflects the co-solvent behavior described before and a slight co-solubility between PMD and water. As it can be observed in Graph. 3.2, the maximum water incorporated in the system to achieve 20% w/w of PMD in the monophasic domain was reached at 42% w/w for 38% w/w of isopropanol (composition corresponding to the point A). In order to increase our knowledge on this solution, Dynamic Light Scattering were performed on the points A and B. Point B represents a pure hydro-alcoholic solution including 38% isopropanol and 62 % w/w water. This solution was chosen in order to detect a hypothetic change of the structure of the formulation in the presence of PMD.

Graph. 3.3 shows the dynamic light scattering autocorrelation functions of the solutions denoted to "A" and "B". There is one typical deviating correlation function for "A" corresponding to the formation of structured aggregates. This system is described (see Introduction) as a "surfactantless microemulsion". The other curve represents the hydro-

alcoholic solution and no established correlation function was found. However, the shape of this curve seems to represent a beginning of spontaneous nanostructures in the solution.

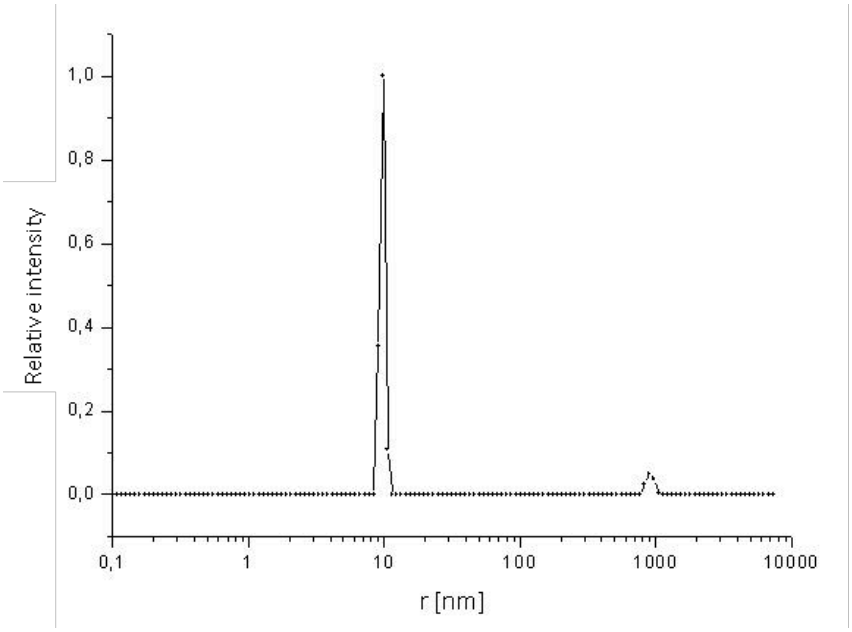


Graph. 3.2: Ternary phase diagram at 25 °C for the three components system PMD/isopropanol/water. Compositions are in weight ratio. L is the domain of existence of the clear and monophasic solutions. N is the domain of existence of the biphasic solutions.



Graph. 3.3: DLS autocorrelation function of a surfactantless microemulsion (point A: 20% PMD, 38% isopropanol, 42% water) (light grey line), and of a hydro-alcoholic solution (point B: 38% isopropanol, 62% water) (dark grey line) measured at 25 °C and at a detection angle of 90.028°.

The distribution function calculated from the DLS autocorrelation function for the surfactantless microemulsion (point A) is shown on Graph. 3.4 A monodisperse distribution is found. The droplet size and the polydispersity are respectively 10.5 nm and 0.106. This kind of microemulsion was rarely reported in the scientific literature[109]. As can be observed in Fig. 3.2, isopropanol can be considered as an effective "hydrotrope" able to render soluble self-assembled molecules of PMD in water[110]. It can be noted that the initial solution of isopropanol in water (point C) on the path of PMD solubilisation showed no defined structure by DLS.



Graph. 3.4: Distribution function calculated from the DLS autocorrelation function of a surfactantless microemulsion (point A: 20% PMD, 38% isopropanol, 42% water).

The lasting protection of the surfactantless microemulsion on volunteers was investigated on a bio-assay. This formulation composed of 20% w/w PMD, 38% isopropanol, 42% water (point A) showed more or less the same time protection of 315 minutes as the alcoholic solution (20% PMD/ 80% isopropanol). This value was within the standard deviation found for the alcoholic solution and reflected the fluctuation recorded while using mosquitoes as an instrument of analysis.

3.3.3. Addition of a surfactant in the "regular microemulsion": Cremophor® RH40

The purpose of this part was to decrease the quantity of isopropanol in the formulation. At first, the aim was to avoid any kind of irritation of the skin due to a large concentration of alcohol on it. Then, limiting the concentration of isopropanol might reduce the penetration of the formulation through the upper skin layers (Stratum Corneum). Short chain alcohols are well known to enhance the penetration of cosmetics through the skin[111-113]. Highest is the penetration and lowest is the lasting protection. At last, this concentration may decrease odor interactions with the active repellent substance (PMD) to get the highest effect possible on the mosquitoes. The addition of a surfactant was done in that direction and Cremophor® RH40 was integrated in the formulation. PEG-40 Hydrogenated Castor oil is one of the main surface active components in pharmaceutical and cosmetic formulations[114]. This substance is a non-ionic solubilizer and emulsifier agent and has a HBL between 14 and 16. Non-ionic surfactants are known to be less irritant compared to the ionic ones for the skin and are widely used for personal care formulations. This component forms clear solutions in water and alcohol and is especially known to get high solubilisation properties with essential oils and perfumes[115]. Besides, Cremophor® RH40 has very little odor and should not interact with the active repellent.

Several solutions were prepared with the integration of PEG-40 Hydrogenated Castor oil at different concentrations in the surfactantless microemulsion (point A). One solution was selected with the lowest quantity of surfactant and alcohol inside: 20% w/w PMD, 13% Cremophor® RH40, 35% Isopropanol and 32% Water. However, with this formulation, a reduction of only 3% of alcohol could be achieved and even the amount of water had to be reduced significantly (from 42% to 32% w/w compare to the formulation A).

Graph. 3.5 represents the dynamic light scattering autocorrelation function of the new formulation. The curve shows the presence of structured aggregates in the selected medium. The distribution function calculated form the DLS autocorrelation (figure not shown) defines a monodisperse distribution with a droplet size of 12.31 nm. Compared to the hydro-alcoholic solution corresponding to point "B", the size of the particles has

slightly increased. The polydispersity index is 0.459. This value might be due to the presence of nano-objects of different shapes in the solution.

3.3.4. Incorporation of a co-surfactant in the microemulsion: 2-ethyl-1,3-hexanediol

A co-surfactant was added to reduce the concentration of Cremophor® RH40. 2-ethyl-1,3-hexanediol (EHD) was chosen for its repellent properties on the mosquitoes and its co-surfactant capacity[116]. This diol was used in the past as the main insect repellent active before the discovery of DEET by the U.S. army in 1946[117,118]. In the past, studies have been investigated on a synergism between DEET and 2-ethyl-1,3-hexanediol, which has the property to enhance the repellent efficacy of DEET on the mosquitoes[119]. This molecule, which is odorless, soluble in water, with a low vapor pressure, presented ideal properties to be integrated in the formulation. Besides, EHD is currently used in several industrial applications especially in pharmaceutics and cosmetics as a solvent or co-surfactant. In human studies, EHD produces weak primary and cumulative irritation in skin and is a weak skin sensitizer[120].

Several solutions were prepared varying the ratios between Cremophor® RH40 and 2-ethyl-1,3-hexanediol. A 2/1 w/w ratio between the surfactant and the co-surfactant allows a great reduction of the quantity of solubilizer. Only 4% of the couple of actives are then necessary to render soluble 20% w/w PMD. A new formulation composed of 20% w/w PMD, 35% Isopropanol, 4% surfactants (2.7% Cremophor® RH40, 1.3% 2-ethyl-1,3-hexanediol) and 41% Water was proposed.

The corresponding dynamic light scattering autocorrelation function is also given in Graph. 3.5. The curve shows the presence of structured aggregates in this medium. The calculated distribution function (figure not shown) suggests a monodisperse distribution with a droplet size of 14.38 nm. The polydispersity index was 0.112 showing quasi monodisperse nanostructures. The addition of 2-ethyl-1,3-hexanediol in the solution reduced the dispersity. This fact was certainly related to the co-surfactant behaviour of EHD.

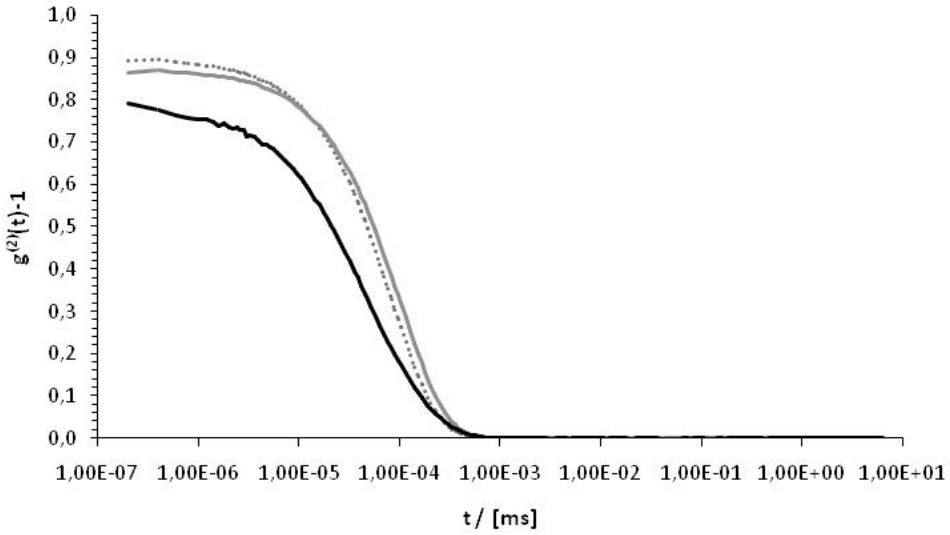
3.3.5. Addition of a second surfactant in the system: Texapon® N70

Based upon the results previously found, a second surfactant was considered to decrease the concentration of isopropanol in the solution. Texapon® N70 (sodium laureth sulphate) was used in the formulation. Sodium laureth sulphate is a detergent and a surfactant found in many personal care products (soap, shampoo etc.)[121]. In previous studies, Texapon® N70 was discovered to have a good synergic effect on perfume molecules solubilisation in association with Cremophor® RH40[122]. This surfactant is also odorless and able to stabilize formulations on a wide range of temperatures. Besides, Texapon® N70 suppresses the clouding of non-ionic surfactants and exhibits a low Krafft point.

Several solutions were prepared by adjusting the ratios between the system "Cremophor® RH40: 2-ethyl-1,3-hexanediol (2:1)" and Texapon® N70 in the solution. Additionally, the new system of surfactants and co-surfactant was introduced in the formulation with different ratios by reducing the amount of isopropanol in favor of water. The concentration of PMD remained constant. A 1/1 w/w ratio between the first system and the second surfactant was found to render soluble the active. The new surfactant system was: 1 (Cremophor® RH40: 2-ethyl-1,3-hexanediol (2/1)): 1 Texapon® N70.

At the end, the new formulation A was composed of 20% w/w PMD, 28% Isopropanol, 6% surfactants (2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon® N70) and 46% Water. Due to the integration of this second surfactant in the formulation, the concentration of isopropanol was reduced from 35% to 28% w/w by the use of two more percents of surfactants from 4% to 6% w/w.

DLS were carried out to reveal the presence of defined structures in the formulation. Graph. 3.5 shows the autocorrelation function obtained for this formulation. The curve demonstrates the presence of structured aggregates in the medium. The distribution function (figure not shown) calculated from the DLS autocorrelation function defined a monodisperse distribution with a particle size of 17.63 nm. The polydispersity index was 0.137, which suggests roughly monodisperse nanostructures.



Graph. 3.5: DLS autocorrelation function of different formulations composed of:

- 20% w/w PMD, 13% Cremophor® RH40, 35% Isopropanol, 32% Water (dotted line),
- Fomulation A: 20% w/w PMD, 35% Isopropanol, 2.7% Cremophor® RH40, 1.3% 2-ethyl-1,3-hexanediol, 41% Water (light grey line),
- Formulation B: 20% w/w PMD, 28% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon®N70, 46% Water (dark grey line).

Autocorrelation functions measured at 25°C and at a detection angle of 90.028°.

Several cages test bio-assays were performed to screen the protection time of the new formulation on yellow fever mosquitoes. A 20% w/w PMD solution in isopropanol was prepared as a control test. Each volunteer tested both solutions at the same time using one forearm with the new formulation and one forearm with the control solution.

Repellent Formulation	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Reference solution	312	70.68	31.61	224.25	399.75	225	405
Formulation A	339	98.13	43.89	217.15	460.85	255	495
Formulation B	385	39.69	15.00	348.30	421.70	345	465

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16400.000	2	8200.000	1.689	**.220**
Within Groups	67950.000	14	4853.571		
Total	84350.000	16			

Table 3.1: Protection time comparison in a bio-assay using *Aedes aegypti* mosquitoes in cage tests of 3 different solutions:

- Reference solution: 20 % w/w PMD solution in isopropanol,
- Formulation A: 20% w/w PMD, 28% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon®N70, 46% Water,
- Formulation B: 20% w/w PMD, 25% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon® N70, 46% Water, 3% Ethyl (S)-(-) lactate.

As shown on Table 3.1, the complete protection time for a 20% w/w PMD solution in isopropanol is around 312 minutes. With the new formulation (A) a protection time of 340 minutes was reached. Compared to the reference solution (20% w/w PMD in isopropanol), the new formulation provided 30 minutes longer protection until the break-off point was reached. However, protection times observed during the test of the new formulation did not differ significantly from the protection times observed during the cage tests for the reference solution ($p>0.05$, T-Test).

3.3.6. Incorporation of a co-solvent in the microemulsion: Ethyl (S)-(-) lactate

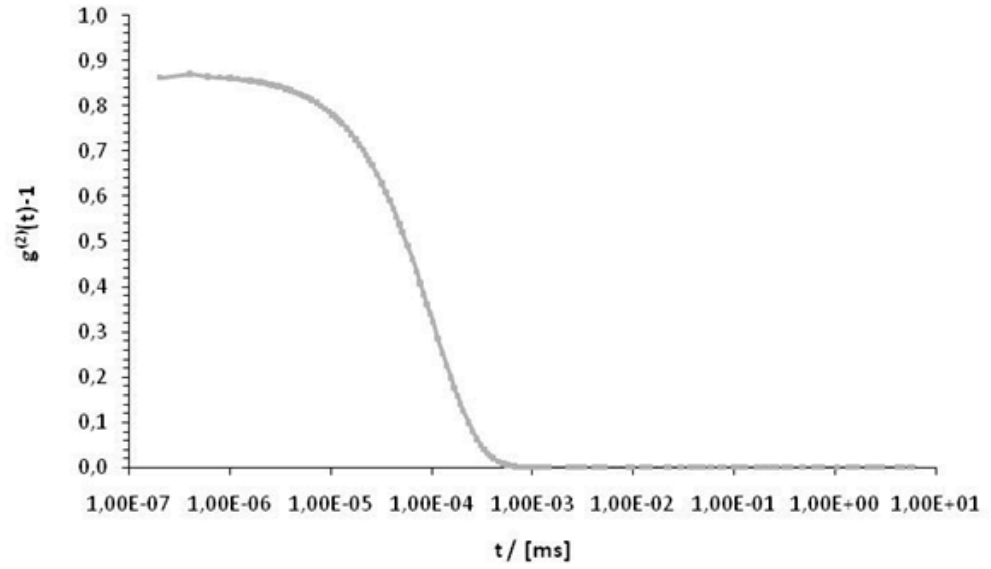
Ethyl (S)-(-) lactate was used to reduce as much as possible the quantity of isopropanol in the formulation. This component has a lower tendency to penetrate through the skin than isopropanol. Lactate esters are non-toxic, biodegradable, and have excellent solvent properties that can replace more toxic solvents for a wide range of industrial and consumer uses[105]. Ethyl (S)-(-) lactate is a common cosmetic ingredient used in fragrances and pharmaceutical preparations with a low toxicity and a slight mild odor. It is soluble in water, in alcohol and paraffin oils. Ethyl (S)-(-) lactate was added in the formulation at different concentrations.

Different cage tests were performed as a function of lactate ester concentration introduced in the system. Three solutions were prepared composed of 3%, 5% and 10% w/w of Ethyl (S)-(-) lactate and the rest of the chemicals previously used. For instance, with a 5 % w/w of Lactate ester, the formulation was composed of 20% w/w PMD, 23% Isopropanol, 6% surfactants, 5% Ethyl (S)-(-) lactate and 46% Water. Surfactant system: 1 (Cremophor® RH40: 2-ethyl-1,3-hexanediol (2/1)): 1 Texapon® N70.

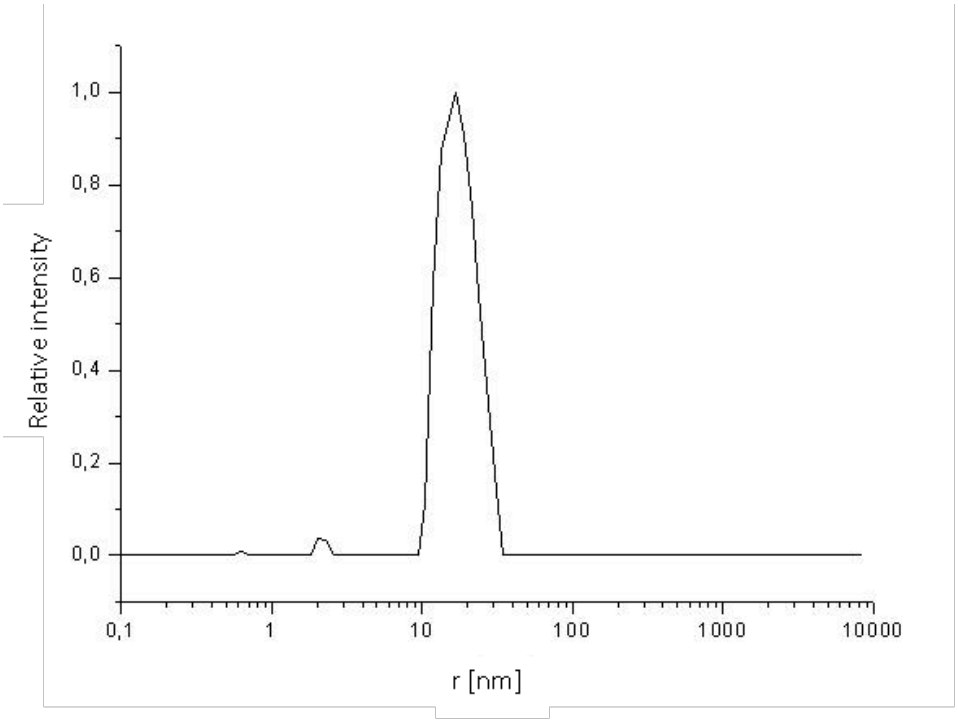
Without Lactate ester, the protection time was around 340 minutes while it lasted 45 minutes longer with 3% w/w Ethyl (S)-(-) lactate and around 20 minutes more with 5% or 10% w/w. As shown on table 3.1, a 3% w/w Ethyl (S)-(-) lactate concentration in the formulation (B) shows a protection time increase of 60 minutes to a mean of 385 minutes of complete protection against *Aedes aegypti* mosquitoes compared to the hydro-alcoholic solution composed of 20% w/w PMD in isopropanol. Statistical analyses were done on the formulation containing 3% Ethyl (S)-(-) lactate. The differences in the protection times between the reference solution and the formulation B containing 3% Ethyl(S)-(-) lactate was significant ($p<0.05$, T-Test).

The non-dose effect obtained with ethyl (S)-(-) lactate could be related to a saturation of the olfactory receptors of the mosquitoes. Ethyl (S)-(-) lactate may act as a competitor for lactic acid present on the skin, which is known to be a good attractant for *Aedes aegypti* mosquitoes[123,124]. A complex synergistic behaviour between PMD, 2-ethyl-1,3-hexanediol and Ethyl (S)-(-) lactate might also be conceivable.

Graph. 3.6 shows the autocorrelation function found by DLS for a final formulation B composed of 20% w/w PMD, 25% Isopropanol, 6% surfactants, 3% Ethyl (S)-(-) lactate and 46% Water. Surfactant system: 1 (Cremophor® RH40: 2-ethyl1,3-hexanediol (2/1)): 1 Texapon® N70 and 3% ethyl (S)-(-) lactate. The curve represents the autocorrelation function corresponding to a structured medium with the presence of nano-aggregates. The distribution function was calculated by the autocorrelation function and as shown on Graph. 3.7 a monodisperse distribution was found with a droplet size of 16.87 nm. The polydispersity index was 0.134 corresponding to quasi monodisperse aggregates in the system.



Graph. 3.6: DLS autocorrelation function of the final formulation B composed of 20% w/w PMD, 25% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon® N70, 46% Water, 3% Ethyl (S)-(-) lactate measured at 25°C and at a detection angle of 90.028°.



Graph. 3.7: Distribution function calculated from the DLS autocorrelation function of the final formulation B composed of 20% w/w PMD, 25% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon® N70, 46% Water, 3% Ethyl (S)-(-) lactate.

3.4. Conclusion

The study confirms the high repellent properties of PMD and the possibility to use this molecule as a natural alternative to synthetic compounds like DEET. The repellent activity can be even maintained in complex formulations like microemulsions. With adapted choices of different additives, an extension of the protection time can be obtained. The protection time can reach an average of 385 minutes using 2-ethyl-1,3-hexanediol as a co-surfactant and Ethyl (S)-(-) lactate as a co-solvent. However, the reason for this extended protection time remains unclear and may be induced by the integration of these two additives as well as the reduction of isopropanol in the formulation.

Chapter 4

FORMULATION, DEVELOPMENT AND MOSQUITO REPELLENT ACTIVITY OF A SKIN LOTION BASED ON *PARA*-MENTHANE-3,8-DIOL

4.1. Abstract

The present study aimed to develop and evaluate a new mosquito repellent lotion (O/W) containing long-lasting *para*-Menthane-3,8-diol (PMD) active. The preliminary organoleptic parameters consisting of physical appearance, color, odor and pH were investigated. The pre-formulated lotions were stored at 21°C±1°C and 40°C±0.1°C for a period of 14 days to predict the stability. The microbiological activity of the lotions was also evaluated with *Staphylococcus Aureus* and *Pseudomonas Aeruginosa* bacteria to comply with microbial limits according to the European Pharmacopoeia. The final PMD lotion was compared to a 20% PMD alcoholic solution and to the manufactured Autan® Protection Plus lotion from SC Johnson (KBR 3023 as repellent active) as controls. The new lotion was proved to exhibit a high repellent activity against *Aedes aegypti* mosquitoes with a mean protection of 480 min compared to 300 min with the alcoholic PMD reference (ANOVA - $p \leq 0.05$) and 448 min for the Autan® product with no significant difference.

4.2. Introduction

Mosquitoes and the diseases they spread have been responsible for killing more people than all the wars in history. Even today, mosquitoes transmitting malaria kill around 440 000 people and infect another 220 million or more every year[125]. To anticipate any kind of epidemic diseases or to avoid any discomfort provided by the mosquitoes, a personal protection is highly recommended[126-128]. Repellent products are the first line of self-defense and few molecules provide some high degree of protection against mosquito bites.

KBR 3023 (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester) has become a standard active by gradually replacing DEET (*N,N*-diethyl-*m*-methylbenzamide) in repellent alternatives with the same efficacy but without the same controversial concerns (blisters and burning sensations, neurological damages)[129-132]. The oil of lemon eucalyptus (OLE), which is the refined version of pure lemon eucalyptus oil has a long history of use but only recently became important as a commercial repellent with long-lasting protection properties against mosquitoes[62,82,133,134]. The essential oil of the lemon consists of 80% citronellal and then is converted into cis- and trans-isomers of *para*-Menthane-3,8-diol (PMD). This process naturally occurs as the leaves of the plant age. Pure PMD could also be synthesized for commercial production from synthetic citronellal[75].

The growing demand of repellents and the consumers interest for more efficient, safe and eco-friendly products drive the industrials to design new formulations[135]. The majority of the marketed repellents are developed with alcohol-based systems, which enhance the penetration of the active through the skin layers and thus reduce the protection time and promotes epidermal irritations[109,136,137].

The present study was undertaken to develop a new repellent oil-in-water (O/W) emulsion containing pure *para*-Menthane-3,8-diol. The oil droplets of PMD are dispersed in the aqueous phase and suitable emulsifiers were used to prevent any macroscopic phase separation[138]. The choice of the components was preferably made on criteria of biological origin to achieve the requirements of the European label COSMOS-standard[139].

COSMOS-certified products are produced to the highest standards for organic and natural cosmetics, are safe, effective and good to use.

Additionally, physical and microbiological stability tests were performed by using a suitable preservatives system. The challenge tests were designed to measure the level of biological activity of the preservative system in the repellent formulations[140,141]. Preservative efficacy test includes artificial contamination of a formulation with a predetermined number of microorganisms followed by periodic removal of samples at fixed time intervals which, after recovery in suitable media, are used for the viable count of the microorganisms present in the formulation[142-145]. The organisms specified for used in the tests are intended to be representative of those that might be expected to be found in the environment in which the preparation is manufactured, stored and used. Manufacturers usually test the ability of preparations to maintain minimum microbial growth by deliberately inoculating the final product with a suitable microorganism such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans, Aspergillus brasiliensis.* An ideal preservative should has a broad spectrum of activity against microorganisms and is compatible with different ingredients of a product and its packaging[146]. Preservatives should be effective at low concentration against all possible microorganisms, stable over the range of pH values[147,148] and non-toxic in nature.

Finally, the protection activity of the O/W repellent emulsion was investigated against *Aedes aegypti* mosquitoes for a potential industrial application and compared to the Autan® Protection Plus lotion.

4.3. Experimental procedures

4.3.1. Formulation and characterization

Chemicals 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Taksago, France). Carbopol 981 NF (Lubrizol, Germany) was used as received. Sodium Benzoate/Potassium Sorbate (euxyl® K 712*) was purchased from Schülke (Germany). Isoamyl Laurate (Dermofeel®sensolv*), Sodium Phytate

(Dermofeel®-PA3*), Glyceryl Stearate Citrate/Cetearyl Alcohol/ Glyceryl Caprilate (Symbio®muls GC*), Tocopherol/Helianthus Annuus (Sunflower) Seed Oil (Dermofeel® Toco 70 non GMO*) (Dr. Streatmans, Germany) were used as received. Stearyl Alcohol (Lanette®18)*, Glyceol*, triethanolamine, citronellol* and vanillin* were supplied by Sigma-Aldrich (Germany). Autan® Protection Plus (SC Johnson) was purchased from a local drugstore. *Ecocert certified

O/W repellent lotion preparation The formulation components used are listed in Table 4.1 Numerous trials were investigated to provide the best lotion in terms of organoleptically and physically properties.

Oil in water emulsion of 20% PMD was formulated. Oil phase (phase A) and water phase (phase B) were prepared separately by mixing the ingredients at 40°C for 30 min. Carbopol was added slowly to phase B under vigorous stirring. Phase B was dissolved in phase A under mixing. The emulsifier was added to the mixture and mixed until complete dissolution of the pellets. The mixture was then homogenised using an Ultra Turax at 6000 rpm for 2 min. The formulation was cooled down at room temperature before the addition of the preservative and the perfume. pH was adjusted to 5.5-6 using triethanolamine.

Physical Analysis The lotions were analyzed organoleptically (color, odor and appearance) and physically (lotioning and phase separation).

pH determination The pH value of various emulsions stored at different conditions was determined using a digital pH Meter.

Stability tests The tests were performed at 21±2°C and 40±0.1°C (Cytoperm Heraeus incubator) for 0, 2, 7, 14 days.

Centrifugation Tests The formulations were centrifuged in a Megafuge 1.0, Heraeus sepatech at 3000 rpm for 45 minutes.

Microscopy The particle size of the lotion was observed with a microscope NIKON Eclipse E400 and using a JVC camera. Samples were observed at 50x magnification.

Rheology The rheological behaviour measurements were performed on a CVO 120 High Resolution rheometer (Bohlin-Malvern instrument, United Kingdom) and a cone-plate sensor with a diameter of 40 mm and a cone angle of 4°. Both shear stress and viscosity are plotted against the shear rate and the viscosity of the lotions was compared for a shear rate of $200s^{-1}$.

Phase	Trade name	Properties	Chemical name	Formulation % (w/w)
Oil phase	Coolact® 38D	Repellent active ingredient	*para*-Menthane-3,8-diol	20
	Dermofeel® sensolv	Emollient	Isoamyl Laurate	6
	Lanette® 18	Emollient	Stearyl Alcohol	2
	Dermofeel® Toco 70 non GMO	Antioxydant	Tocopherol, Helianthus Annuus (Sunflower) Seed Oil	0.5
Water phase		Solvent	Distilled Water	61.98
	Glycerol 87 % p. A.	Humectant	Glycerol	5
	Symbio®muls GC	Chelating agent	Sodium Phytate, Aqua, Alcohol	3
	Carbopol 981	Rheology modifier	Acrylic Acid Polymer	0.5
Emulsifier	Dermofeel PA-3	Emulsifier blend for O/W	Glyceryl Stearate Citrate, Cetearyl Alcohol, Glyceryl Caprylate	0.1
Additives	Triethanolamine	pH adjuster	Triethanolamine	0.75
	Vanillin	Repellent booster	Vanillin	0.1
	Citronellol	Perfume	Citronellol	0.07
	Euxyl® K 712	Preservative	Aqua, Sodium Benzoate, Potassium Sorbate	

Table 4.1: Trade name, functions, chemical name and concentration of components used in the final repellent lotion.

4.3.2. Microbiological tests

Challenged Microorganisms Microbiological challenge tests according to the European Pharmacopoeia[141] have been performed with the Gram-positive bacteria *Staphylococcus Aureus* DSM 799 and the Gram-negative bacteria *Pseudomonas Aeruginosa* DSM 1128 purchased from DSMZ, Germany. The collection strains are stored at -80°C in the laboratory under conditions laid out in standard EN 12353.

Media Dehydrated CASO Agar medium from Sigma-Aldrich (Germany) were dissolved in Mili-Q water and were sterilized in the autoclave at 121°C for 15 minutes.

Protocol of Challenge tests

1. 500 µL of the frozen culture were washed and centrifuged at 3000 rpm for 7 min two times with the medium and two times with a solution of NaCl (9g/L).
2. 40 mL of NaCl solution were added to the bacteria.
3. The absorbance at 600 nm was measured with a photometer for two solutions:
   - 100 µL of bacteria suspension in 900 µL of water,
   - 100 µL of NaCl solution in 900 µL of water.

A calibration (number of Colony-Forming Unit (CFU)/mL of inoculum against the optic density) has been previously done for each types of bacteria.

4. The appropriate quantity of the bacteria solution was replaced by the same amount of NaCl solution in order to have a bacteria's suspension of 10^8 CFU/mL.

An optic density of 0.9 correspond to 10^8 *S. Aureus* CFU/mL.

An optic density of 1.83 correspond to 10^8 *P. aeruginosa* CFU/mL.

5. The bacteria's suspension was mixed with the lotion in order to have 10^6 CFU/mL of the mixture. The percentage of bacteria in the mixture was less than 1%.
6. Samples were stored, and the following steps were performed after 0, 2, 7, 14 days:
   - Inoculum were diluted in the media: 1:10, 1:100, 1:1000,
   - 30 µL of each dilution was spread on tryptic soy agar plates with a Drigalski spatula,
   - Plates are stored at 35°C for 1-2 days and then the number of colonies on each plate was recorded. Only plates containing a number of germs between 15 and 300 were counted.

A blank solution containing 5% of glycerin in water was as well inoculated with these two bacteria following the previous protocol.

4.3.3. Mosquito repellent study

Insects Strain of *Aedes aegypti* mosquitoes from Bayer AG were reared according to the standard protocol at 27°C, a relative humidity of 60–80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in H2O filled with a 1:1 mixture of tap- and deionised H_2O, and fed with fishfood flakes (Tetra Min®). Before hatching the pupae were transferred to a

cage (40 x 30 x 20 cm) and provided with sugar soln. (10% dextrose). Mosquitoes at an age of 9-14 days were used for the tests.

Volunteers Five human volunteers aged between 20 and 26 participated in the mosquito cage test bioassay. No abnormal allergic reaction after application of the formulations was observed.

Laboratory tests Human skin tests were conducted as described below.

Application of repellents: The skin of the forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% iPrOH and then dried with a paper towel. An area larger than the test window was marked on the skin with a metal template. According to the United States EPA, 1 g of pump spray is applied per 600 cm² skin. The marked area had a size of 98 cm²; therefore 0.2 g were applied to the test area. The test substance was applied using a pipette with disposable tips, for each test person a new tip was used. The test substance was spread evenly on the skin of the forearm wearing a latex glove.

Exposure to mosquitoes: Thirty mosquitoes were placed in a test cage that was fitted with a test window in the floor of the cage. The test window was closed by a metal slide and was opened by inserting a metal frame for the exposure of the treated quadratic (98 cm²) skin area (ventral) and untreated area (back) of the volunteer´s forearms. This method was designed so that each volunteer served as his own control.

Zero control: Zero control is the untreated back of the forearm of the test person. A window frame with mosquito net is used to keep probing mosquito from successfully taking a blood meal. Biting pressure must exceed 10 probings in 30 s. After 30 s and more than 10 probings the mosquitoes were considered as active and suitable for the experiment.

Test proper: Each test person was assigned to one test cage. Between two tests, a special air ventilation system was attached to the cage in order to prevent any accumulation of odors and active substances in the cage. The treated skin was exposed to the mosquitoes for a testing time of 2 min. In this time, the number of landings and bitings on the treated skin was noted and compared to the untreated control skin.

Determination of duration of protection: As far as there is no official protocol in EU, tests were based on the EPA draft guideline OPPTS 810.3700 (EPA, 2000). The criteria to

define a complete protection time were dependent on the zero control. A 15 minutes margin of error was attributed on every result.

Preparation of the tested solutions The lotion described in Table 4.1 was prepared and 0.17g were applied on the forearm of each human volunteer. Additionally, a 20% (w/w) solution of PMD in ethanol was used as well as the Autan® Protection Plus lotion (20% KBR 3023) as controls.

4.3.4. Statistical analysis

Data reported time of protection were subjected to an analysis of variance (ANOVA) and Post-Hoc T-test ($P<0.05$) using the software IBM SPSS Statistics (version 22 for windows).

4.4. Results and discussion

4.4.1. General properties

Different pre-formulations were prepared using multiple components at different concentrations and were submitted to analysis. Several emulsions were selected to additional studies in order to obtain a final repellent lotion with appropriate properties.

Physical properties

The stability studies of various parameters for the final formulation like visual appearance, texture, color, odor and pH showed that there was no significant variation after 14 days of the study period. The results are summarized in table 4.2. The repellent formulation composed of 20% PMD shows no redness, edema, inflammation and irritation on human skin during the period.

DAYS / TESTS	Repellent Lotion (20% PMD)			
	0	2	7	14
Physical appearance	Lotion	Lotion	Lotion	Lotion
Texture	Soft / non greasy or sticky	Soft / non greasy or sticky	Soft / non greasy or sticky	Soft / non greasy or sticky
colour	Snow white	White	White	White
Odour	Faint mint / vanilla	slight odour of vanilla	slight odour of vanilla	slight odour of vanilla
pH value	5.7	5.6	5.6	5.5
Thermal stability	ok	ok	ok	ok
Degradation of product	nil	nil	nil	nil

Table 4.2: Stability Studies were conducted at 21 ± 1°C and 40 ±0.1°C for the final formulation.

Phase Separation

Emulsions are thermodynamically unstable and droplets merge with each other to produce big droplets and increase the coalescence rate. Emulsions can deteriorate by creaming, flocculation, Ostwald ripening, or partial coalescence, which leads to coalescence[149]. Coalescence is one of the possible mechanisms of destruction of emulsions, which occur when the energy of adhesion between two droplets is larger than the turbulent energy causing dispersion[150]. For several pre-formulations, slight separations were observed at 21°C or 40°C on 14 days of observation. Phase separation may be attributed to the movement of small number of surfactant molecules from interface to the surface. The final lotion was stable in all storage conditions.

Centrifugation Tests

No phase separation was observed after centrifugation with the final lotion in any of the different storage conditions i.e., 21 and 40 ± 2°C up to 14 days of observation. This indicated that the emulsions were stable at least over two weeks.

Microscopic studies

Microscopic and globule size analysis is a quite useful direct method to assure formation of O/W emulsions as well as to predict stability of multiple globules over time. Droplet size measurements are a good indicator of the formulation stability.

Microscopic analysis was performed after vigorous centrifugation to determine whether multiple globules are resistant or not. An emulsion is characterized by a droplet size between 1 and 10 μm.

Results of the investigation reveal that the mean globule size of multiple droplets was recorded in the range of 8 μm (see Figure 4.1). An increase in the polydispersity and the droplet size over time or temperature characterize the non-stability of lotions. The droplet size of globules did not change over the test period.

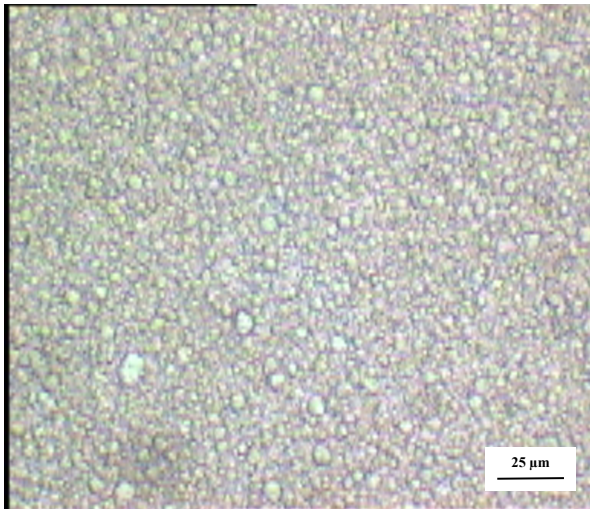
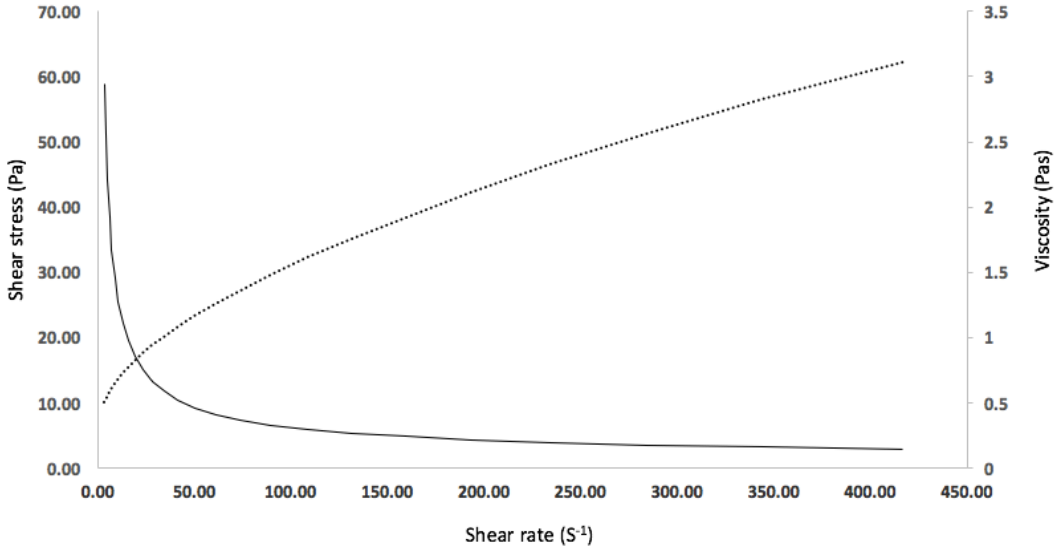


Figure 4.1: Photomicrograph of the final emulsion kept at room temperature.

Rheology experiments

The consistency of the multiple pre-formulated emulsions, which is a key factor for skin repellent applications, were evaluated using rheological methods. Most emulsions are non-Newtonian and show pseudo-plastic flow. The shear stress as well as the viscosity are plotted against the shear rate. The result performed on the final formulation showed that the viscosity decreases with increase of the shear rate (see Graph. 4.1). This behavior is common for non-Newtonian flow and shear thinning behavior like lotions[151]. Viscosity values are compared at a shear rate of 200 s^{-1} on the ascendant slope. Mean apparent viscosity for the final repellent lotion was 20.000 mPas.



Graph. 4.1: Viscosity of the final repellent formulation as a function of shear rate.

4.4.2. Microbiological tests

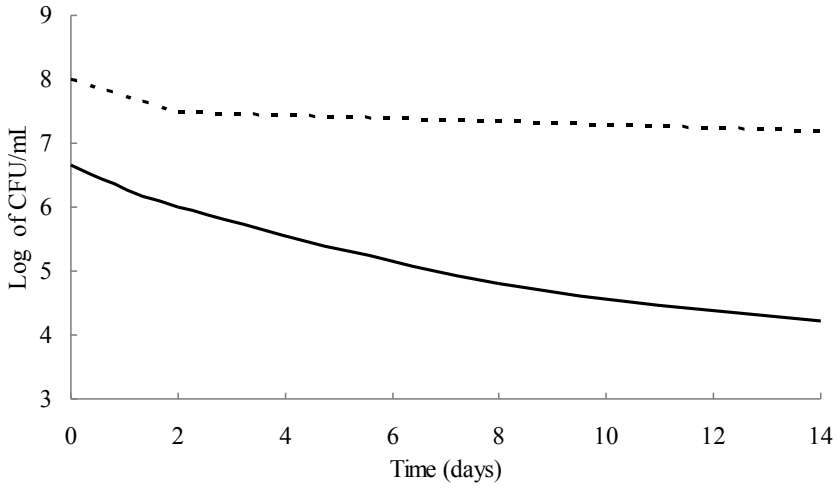
The antibacterial activity of the selected lotion (See Table 4.1) with varying amounts of preservative euxyl® K 712 (Sodium benzoate/Potassium sorbate/aqua) 0, 0.5 1, 1.5 % (w/w) was studied against *P. Aeruginosa* and *S. aureus*. This preservative was developed for leave-on cosmetic formulations with a skin-friendly pH value of up to 5.5. It has a broad, balanced spectrum of effect against bacteria, yeasts and mold fungi. It has a good compatibility with surfactants, is fully soluble in water and can be integrated during a cold process. The level of contamination was monitored on 0, 2, 7 and 14 days by counting the colony forming units (CFU) of microorganisms by pour-plate method subsequent to the inoculation. From the calculated concentration of CFU/ml present at the start of the test (0 day), the log reduction in CFU/ml for each microorganism at the different time intervals (2, 7, and 14 days) were calculated.

Blank formulation (Control)

5% glycerine in water was used as a control in this experimental study. The formulation was challenged with the tested bacteria in order to verify the protocol test. For skin sprayable emulsions, the criteria of efficiency recommended for preservatives on bacteria are:

- A reduction of the initial population of bacteria of 2 log in 48h;
- A reduction of the initial population of bacteria of 3 log in 7 days;
- No increase of the population after the 7th day.

The heavy growth of all tested bacteria was observed on 0 day and growth was slightly declined on 2, 7 and 14 days as shown on Graph. 4.2.



Graph. 4.2: Plots of the log of CFU/mL for a solution of 5% glycerine in water inoculated with *S. Aureus* (straight line) and *P. Aeruginosa* (dash line).

At day 2 the reduction of the initial population of both bacteria is lower than 2 log meaning that the first criterion is not respected. This blank formulation needs the use of preservatives to reduce the population of bacteria. The results obtained with the control solution show the efficiency of the protocol used for this challenge test.

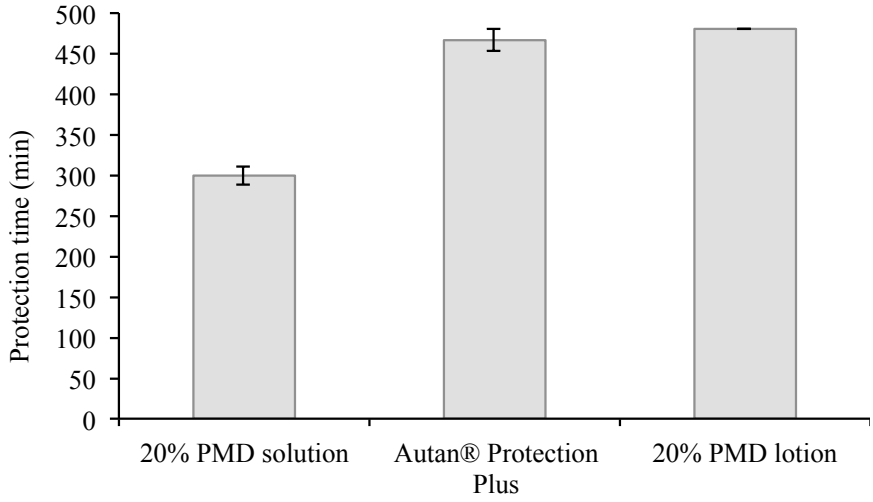
Lotion formulation

The microbial challenge tests of the repellent lotion with different concentrations of preservatives showed no growth with all tested bacteria *S. Aureus* and *P. Aeruginosa.* The Lotions even without preservative did not exhibit a development of colonies after day 2 meaning that preservatives did not have any significant effect. These results might be explained by the presence of PMD in high concentration (20% w/w). Previous studies have shown the efficient antibacterial activity of PMD[152]. Additionally, it has been suggested by Al-Adham *et al.*[153], that the antimicrobial nature of oil-in-water emulsions

may result from the antimicrobial action of the individual components of microemulsion formulations. In particular, many of surfactants and co-surfactants of microemulsions have proven antimicrobial action. Therefore, the addition of preservatives in the present repellent lotion is not necessary.

4.4.3. Mosquito bioassay

The repellent protection activity against mosquitoes of the final lotion as well as with the control samples was determined in a bioassay. Graph. 4.3 represents the protection time until break off of the three tested repellents.



Graph. 4.3: Repellent activity of the final formulation on human volunteers with *Aedes aegypti* mosquitoes.

The PMD control solution provided a mean protection of 300 minutes, which is in the range of previous results observed in the same conditions (See Chapter 3). The Autan® lotion displayed a high activity with 448 minutes, which corresponds to the 8 hours protection claimed by the manufacturer. Finally, the new PMD lotion is highly efficient with 480 minutes of protection against *Aedes aegypti* mosquitoes. Analysis of variance (ANOVA - $P<0.05$, Table 4.3) proved that the final PMD lotion has a statistically higher repellent action than pure PMD in an alcoholic solution. This may be due to the properties of the lotion itself, which may form a film barrier that prevents any transfer through the upper skin layers. The use of several emollients in the lotion decrease the exchange of

water through the skin (dehydration action), but it also reduces the penetration of any other ingredient. The PMD active may stay on the surface for a long-lasting effect that yields to a greater degree of repellency. Also, no significant difference (P>0.05, Table 4.3) was observed between the manufactured product Autan® Protection Plus and the new lotion with the same amount of repellent active. This result demonstrates that repellent formulations composed of pure PMD are able to achieve the same protection efficiency as standard products with KBR 3023 for instance, by developing suitable formulations without alcohol and by using eco-friendly additives.

Repellent	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
20% PMD solution	300	26.22	11.73	267.44	332.56	265	330
Autan® Protection Plus	467	32.66	13.33	432.39	500.94	400	480
20% PMD lotion	**480**	**0.00**	**0.00**	**480.00**	**480.00**	**480**	**480**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	103041.667	2	51520.833	82.858	**.000**
Within Groups	8083.333	13	621.795		
Total	111125.000	15			

Repellent		Mean Difference (I-J)	Std. Error	Sig.
20% PMD solution	Autan® Protection Plus	-166.667	15.099	.000
	20% PMD lotion	-180	15.771	.000
Autan® Protection Plus	20% PMD solution	166.667	15.099	.000
	20% PMD lotion	-13.333	15.099	.660
20% PMD lotion	**20% PMD solution**	**180**	**15.771**	**.000**
	Autan® Protection Plus	**13.333**	**15.099**	**.660**

Table 4.3: One-way ANOVA T-test (P<0.05) of repellent formulations.

4.5. Conclusion

The present study indicated that the new repellent lotion (O/W emulsion) containing *para*-Menthane-3,8-diol, yields good organoleptic and physical characteristics. No evidence of phase separation and a good consistency during the study period was observed. Furthermore, the final formulation has a pH value of 5.6, which is close to the skin surface pH. Preliminary microbiological assays (with *P. Aeruginosa* and *S. aureus* bacteria) on the final emulsion revealed that the lotion was stable without the addition of preservative. Also, bioassay studies have shown a high level of protection (8 hours) on *Aedes aegypti* mosquitoes with no significant difference with the standard repellent Autan® Protection plus. The new formulated repellent lotion was proved to exhibit

promising properties by PMD as a main active. This might open new opportunities for the development of more efficient, and eco-sourced mosquito repellents, which meets the needs of today's consumers.

Chapter 5

STUDIES ON INCLUSION COMPLEX OF *PARA*-MENTHANE-3,8-DIOL WITH CYCLODEXTRINS. INVESTIGATIONS ON REPELLENT ACTIVITY AGAINST MOSQUITOES

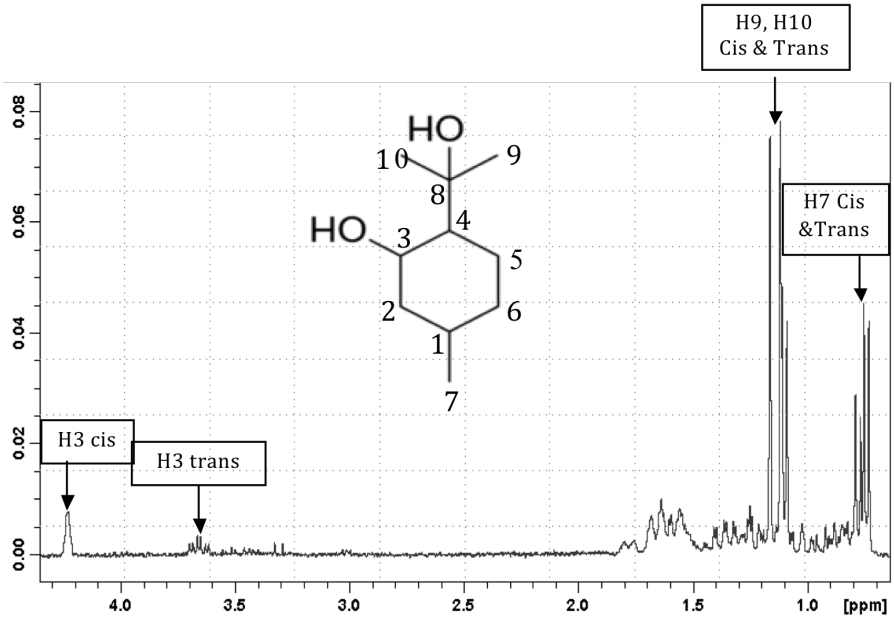
5.1. Abstract

Para-Menthane-3,8-diol (PMD) is a well-known and effective repellent active against mosquitoes. The major challenges in formulating PMD for leave-on products include solubility in water and a control release over time to provide a long-lasting protection. Cyclodextrins (CDs) are represented as a torus-shape with a hydrophilic outer surface and a hydrophobic central cavity, which can encapsulate guest molecules like PMD. Complexation of PMD into β-CD, HP-β-CD and γ-CD was investigated. The stoichiometry as well as the association constant of PMD:CD was determined by ^{1}H NMR spectroscopy. The 1:1 stoichiometry of complexation was established by continuous variation (Job's plot) method and the association constant of the inclusion complex (K_a = 753, 283, 265 M^{-1} for ICs with β-CD, HP-β-CD and γ-CD respectively) was determined by using the Scott's method based on the representation of Benesi and Hildebrand. PMD:CD was confirmed by the shift variation of CD and PMD protons due to the introduction of PMD into the CDs' cavity. The complexation geometry was obtained from 2D ROESY. Finally, the complexation was demonstrated by using Thermogravimetric analysis (TGA). The encapsulation of PMD into CD's cavity

improves the solubility of PMD in water, nevertheless, mosquito repellent tests with *Aedes aegypti* on IC PMD:HP-β-CD did not show any efficient protection.

5.2. Introduction

Para-Menthane-3,8-diol (PMD) is used for its high repellent activity against different mosquito species. PMD is mainly a synthetic mixture of (+)-cis (62%) and (-)-trans (38%) isomers[75]. It was discovered in the 1960's and previous studies have shown that each isomer has different repellent properties. The trans isomer shows a better repellency while the cis has a longer lasting effect. The cis/trans ratio 62/38 provides the highest repellent action on *Aedes aegypti* mosquitoes[70,94]. Graph. 5.1 shows the chemical structure of PMD and its ^{1}H NMR spectra.



Graph. 5.1: ^{1}H NMR spectra of PMD

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six (α-CD), seven (β-CD), eight (γ-CD) or more glucopyranose units linked by α-(1→4) bonds. They are produced as a result of intramolecular transglycosylation reaction from degradation of starch by cyclodextrin glucanotransferase (CGTase) enzyme[154-157]. Cyclodextrins are represented

in Figure 5.1 as a torus-shape with a hydrophilic outer surface and a hydrophobic central cavity.

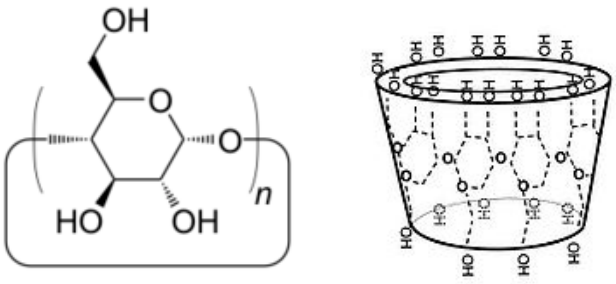
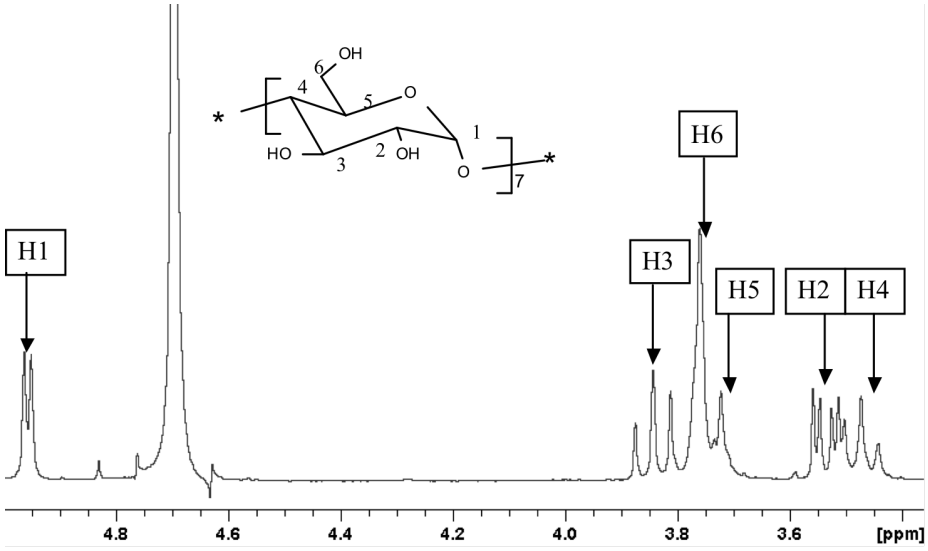


Figure 5.1: Chemical structure of Cyclodextrins and example of the torus structure showing spatial arrangement.



Graph. 5.2: ^{1}H NMR spectra of β-CD.

The main feature of CDs is their ability to form inclusion complexes (IC) with a large range of hydrophobic guest molecules. These ICs are used to improve the guest molecule stability (against UV, oxygen, solubility), to encapsulate unpleasant odors or to provide a slow and long-lasting release of active molecules[158].

The solubility of PMD in aqueous medium is low (0.29 g/l) and its penetration through the upper skin layer via alcoholic base formulations is potentially fast. These two aspects influence drastically the efficacy of PMD as a mosquito' repellent. Complexation of PMD into Cyclodextrins may modify PMD's solubility in water, reduce its penetration through the skin and enable its control release over time.

The IC of PMD into CDs cavity mainly depends on the inner size of the cavity (see Table 5.1). As PMD is a small cyclic molecule, the diameters of CD's cavity have to be considered. Therefore, for the present purpose, IC with β-CD, HP-β-CD (2-Hydroxylpropyl-beta-cyclodextrin) and γ-CD were investigated.

NMR spectroscopy[159-163] and thermogravimetric measurements[164-166] are the most widely used techniques to study CD complexes. This study characterizes the inclusion of PMD in the three different types of CDs. At first, the stoichiometry of the IC PMD:CD was investigated using the well-known continuous variation techniques the so called Job's method. Then the association constant was calculated using the Scott's method following the chemical shift variation of free and complexed CDs protons. In a second part, ROESY NMR measurements and thermogravimetric analysis were performed to confirm the inclusion of PMD in the three different systems. Finally, repellent studies were carried out on *Aedes aegypti* with a solution of IC.

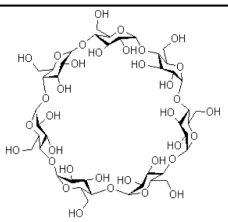
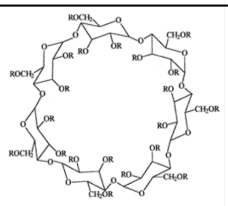
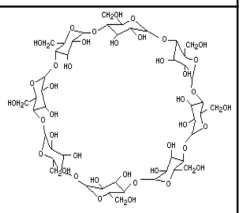
Properties	β-cyclodextrin	HP-β-cyclodextrin	γ-cyclodextrin
formula		 R=$CH_2CH(OH)CH_3$	
number of glucipyranose units	7	7	8
molecular weight (g/mol)	1135	1400	1297
solubility in water at 25°C (g/L)	18.5	2300	232
outer diameter (A)	15.4	15.4	17.5
cavity diameter (A)	6.0-6.5	6,0-6,5	7,5-8,3
Height of torus (A)	7.9	7.9	7.9
cavity volume (A3)	262	262	427

Table 5.1: Main properties of β-CD, HP-β-CD and γ-CD.

5.3. Experimental procedures

5.3.1. Chemicals and Analysis

Products β-CD (98%), HP-β-CD (98%) and γ-CD (98%) (pharma grade) were gently offered by Wacker Specialties (Germany). 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Taksago, France) was used with a specific ratio between the (+)-cis and (-)-trans isomers (62/38) according to the manufacturer. Disodium phosphate (Na_2HPO4; Merck, Germany) and Sodium dihydrogen phosphate (NaH_2PO_4; Merck, Germany) were used as received. Deuterium oxide D_2O was provided by Deutero (Germany).

NMR study ^1H NMR experiments were carried out on a Bruker AVANCE 300 spectrometer (Germany) operating at 300,13 MHz and 22°C equipped with a Ø 5 mm QNP probe with z-gradient coil. The 2D-ROESY[167] (Rotating frame Overhause Effect SpectroscopY) spectra were carried out on a Bruker AVANCE III 600 operating at 600,25MHz and 25°C and equipped with a Ø 5 mm TCI Cryo-Probe (1H, 13C, 31P) with z-gradient. In all experiments, standard NMR tubes (5mm) were used. A phosphate buffer in D_2O (pD=7,2) is used as a solvent for all the experiments.

Determination of the inclusion complex stoichiometry The stoichiometry of the complex was determined by using the well-known continuous variation techniques, the so called Job's method[168,169].
The complex PMD:CD formed has a n: 1 geometry.

$$n\ PMD + CD \rightleftharpoons Complex\ PMD{:}CD$$

The inclusion of PMD into CD is shown by the variations in the chemical shifts of some of the guest and host protons in comparison of the chemical shifts of the same proton in the pure molecules.

$$\Delta\delta = |\delta - \delta'|$$

$$rX=[X] / ([PMD] + [CD]) \text{ with } X= PMD \text{ or } CD$$

Δδ is the chemical shift variation between proton in the inclusion complex and in pure molecules. δ is the chemical shift of the free compounds. δ' is the chemical shift of the proton in the inclusion complex. [X] is the concentration in compound X. According to the Job's method, by plotting Δδ*[X] versus rX, a curve is obtained showing a maximum of rX. This maximum corresponds to the maximum concentration of the complex formed.

Samples for NMR analysis were done by mixing 24h two equimolar (2 mM) solutions of CD and PMD in different ratios (see Table 5.2) (Vtotal = constant). The total concentration of the interacting species in the solution was kept constant ([PMD] + [CD] = 2mmol/L) and the molar fraction rX varies in the range of 0 to 1.

r_{PMD}	[PMD] mmol/L	[CD] mmol/L
0	0	2
0.4	0.8	1.2
0.5	1	1
0.6	1.2	0.8
0.8	1.6	0.4
1	0	2

Table 5.2: Composition of the samples used to determine the stoichiometry of the complex PMD:CD.

Determination of the association constant The association constant (K_a) of the PMD:CD complex in a stoichiometry 1:1 was determined by using the well-known Scott's method, which is a modification of Benesi-Hildebrand[170]equation

$$PMD + CD \rightleftharpoons PMD:CD$$

$$K_a = [PMD:CD]/ ([PMD]*[CD])$$

where [CD] is the concentration of free CD, [PMD] is the concentration of free guest and [PMD:CD] the concentration of the IC.

$$[CD]/ \Delta\delta_{obs} = [CD]/ \Delta\delta_{max} + 1/ (K_a*\Delta\delta_{max})$$

[CD] is the total molar concentration.

$\Delta\delta_{obs}$ is the chemical shift difference observed for the PMD protons in the mixture. $\Delta\delta_{max}$ is the chemical shift difference between the PMD protons in the pure complex. According

to the Scott's method[171], by plotting [CD]/ $\Delta\delta_{obs}$ as a function of [CD], a straight line is obtained. Its slope corresponds to the opposite of the maximum chemical shift variation, 1/ $\Delta\delta$max and the intercept with the vertical axis is 1/ (K_a*$\Delta\delta_{max}$).

The association constant was calculated from the chemical shift variation measured on the complexation of a 1mM solution of PMD with increasing concentration of CD from 2 to 10mM.

Preparation of solid inclusion complex The complexation was prepared by mixing 24h at room temperature an aqueous solution of CDs with PMD. Solid-state complexes are obtained using a freeze-drier Modulyo EF4 with a vacuum pump Pirani from Absolute Vacuum Services Ltd (United Kingdom). Samples were then stored in a desiccator. According to previous studies, the reaction time, the temperature, the solvent, the percentage of CDs and the mass ratio PMD/CD are the main parameters influencing the complexation[172]. All experiments were performed at room temperature. Only HP-β-CD and γ-CD were used. A control experiment (CDs without PMD) was done to compare the different samples; the same conditions were used for each powder.

Thermo-gravimetric measurements CDs and their solid inclusion complexes were subjected to thermo-gravimetric measurements under nitrogen flow[173]. A TGA 7 from Perkin Elmer corp. (USA-Norwalk, CT) was used. A heating program was settled up from 25°C to 400°C. A constant gas flow of 25mL.min^{-1} was set for all the tests. The precision of temperature measurement for the thermobalance is ±1°C. The residual mass was plotted against the temperature.

5.3.2. Mosquitoes bioassay

Insects Strain of *Aedes aegypti* mosquitoes from Bayer AG were reared according to the standard protocol at 27°C, a relative humidity of 60–80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in H2O filled with a 1:1 mixture of tap- and deionised H_2O, and fed with fish food flakes (Tetra Min®). Before hatching, the pupae were transferred to a

cage (40 x 30 x 20 cm) and provided with sugar soln. (10% dextrose). Mosquitoes at an age of 9-14 days were used for the tests.

Volunteers Five human volunteers aged between 20 and 26 participated in the mosquito cage test bioassay. No abnormal allergic reaction after application of the formulations was observed.

Laboratory tests Human skin tests were conducted as described below.
Application of repellents: The skin of the forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% iPrOH and then dried with a paper towel. An area larger than the test window was marked on the skin with a metal template. According to the United States EPA, 1 g of pump spray is applied per 600 cm² skin. The marked area had a size of 98 cm²; therefore 0.2 g were applied to the test area. The test substance was applied using a pipette with disposable tips, for each test person a new tip was used. The test substance was spread evenly on the skin of the forearm wearing a latex glove.

Exposure to mosquitoes: Thirty mosquitoes were placed in a test cage that was fitted with a test window in the floor of the cage. The test window was closed by a metal slide and was opened by inserting a metal frame for the exposure of the treated quadratic (98 cm²) skin area (ventral) and untreated area (back) of the volunteer´s forearms. This method was designed so that each volunteer served as his own control.

Zero control: Zero control is the untreated back of the forearm of the test person. A window frame with mosquito net is used to keep probing mosquito from successfully taking a blood meal. Biting pressure must exceed 10 probings in 30s. After 30s and more than 10 probings the mosquitoes were considered as active and suitable for the experiment.

Test proper: Each test person was assigned to one test cage. Between two tests a special air ventilation system was attached to the cage in order to prevent any accumulation of odours and active substances in the cage. The treated skin was exposed to the mosquitoes for a testing time of 2 min. In this time, the number of landings and bitings on the treated skin was noted and compared to the untreated control skin.

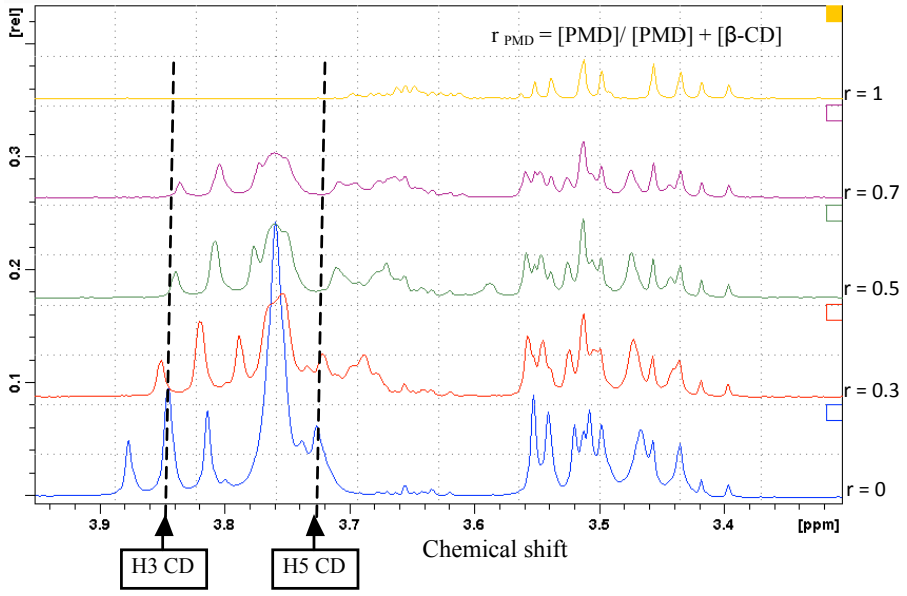
Determination of duration of protection: As far as there is no official protocol in EU, tests were based on the EPA draft guideline OPPTS 810.3700 (EPA, 2000). The criteria to define a complete protection time were dependent on the zero control. A 15 minutes margin of error was attributed on every result.

Preparation of the tested solutions An aqueous solution of PMD:HP-β-CD was prepared using 50 % wt (5% PMD) of the IC. The powder was dissolved slowly in the water phase by a gentle stirring at room temperature. Additionally, a 10% (w/w) PMD solution using ^{i}PrOH as a solvent was used as a control. All solutions were applied on each forearm of the human volunteers.

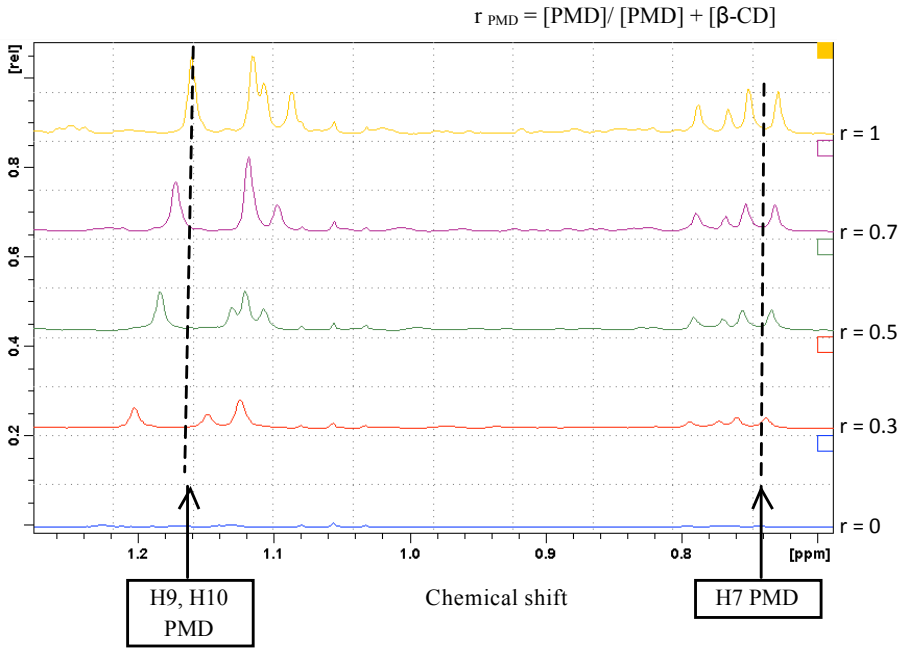
5.4. Results and discussion

5.4.1. Characterization of the inclusion complex PMD:CD in aqueous solutions: example with β-CD

In order to characterize the potential inclusion complex PMD:CD, the chemical shifts variation of the H3 and H5 protons of the CD and H7, H9, H10 protons of PMD. The following plots on Graphs. 5.3 and 5.4 show the main marked variations of β-CD and PMD protons.



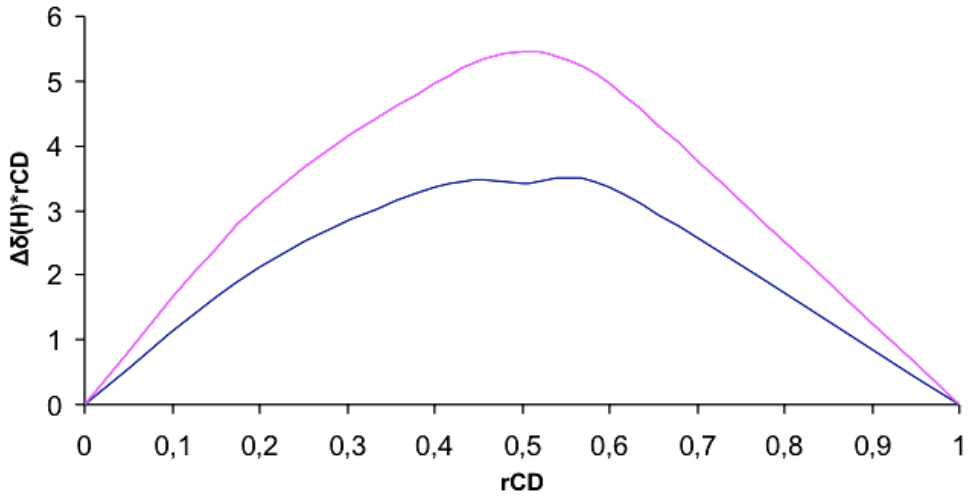
Graph. 5.3: ^{1}H NMR spectra of the inclusion complex PMD:β-CD (CD part).



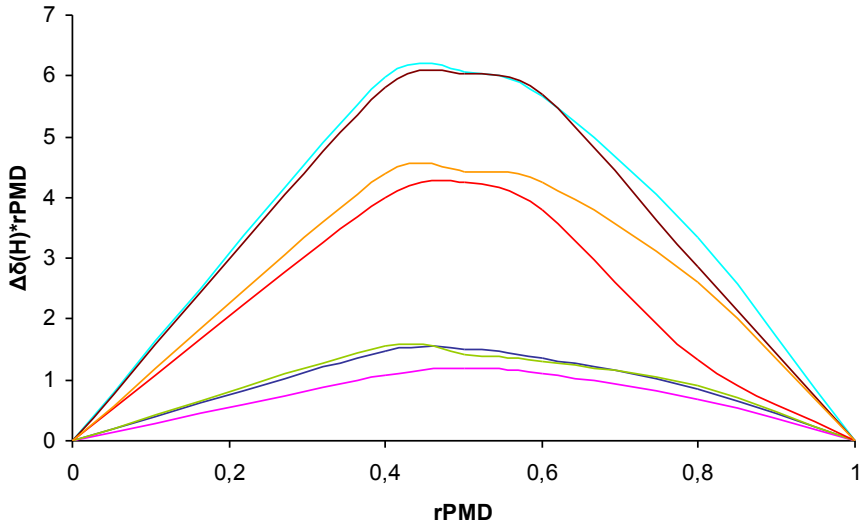
Graph. 5.4: ^{1}H NMR of the inclusion complex PMD:β-CD (PMD part).

Regarding the NMR plots of β-CD protons, H3 and H5 protons located in the central cavity are considerably shifted compared to H4, H6, and H2 protons. Also, PMD protons H9, H10 and H3 seem to be more affected by the complexation and H7 protons suffer from a small shift suggesting that they remain outside the β-CD cavity.

Graphs. 5.5 and 5.6 represent the Job's plot (continuous method) of PMD:β-CD inclusion complex at different concentrations.



Graph. 5.5: Job's curve for β-CD protons H3 (—) and H5 (—).



Graph. 5.6: Job's curves for PMD protons: H3 (—), H7cis (—), H7trans (—), H9cis (—), H9trans (—), H10 cis (—), H10trans (—).

The shape of the curves is not perfectly symmetrical. Nevertheless, it shows a maximum, which does not vary from r= 0.5. This result proves the presence of a complex with a 1:1 stoichiometry in the range of the concentrations observed.

In conclusion, PMD is mainly complexed into CDs' cavity, but also partially adsorbed on the CDs surfaces. Figure 5.2 is a model of complexation between PMD and CD according to the NMR results. However, a slight shift of protons from the outside cavity is also observed, which may be explained by the additional absorption of PMD molecules on the CDs' surface.

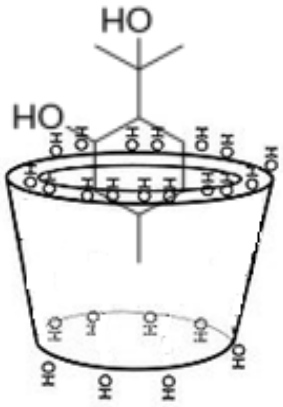
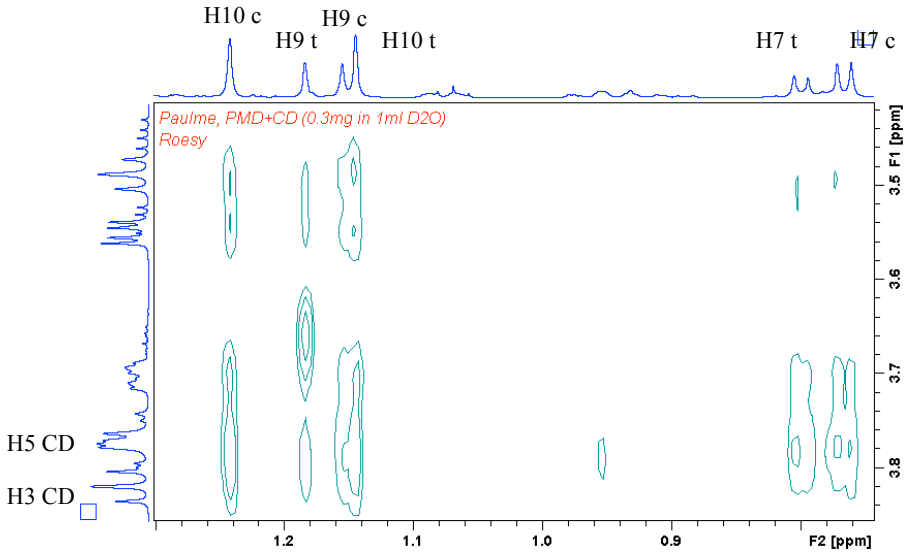


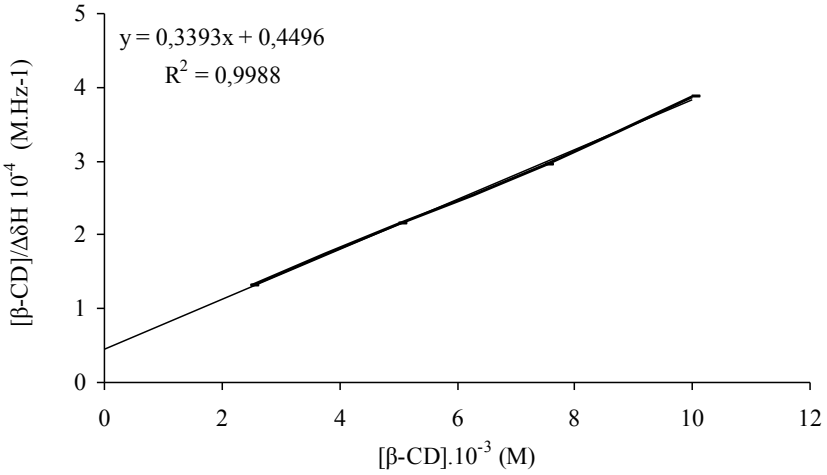
Figure 5.2: Model of the inclusion complex between PMD and β-CD.

To confirm PMD inclusion in β-CD, 2D ROESY experiments were performed. Cross peaks connecting protons of PMD and β-CD were observed in Graph. 5.7. Protons H7, H9, H10 of PMD are connected to the H3 and H5 protons of CDs.



Graph. 5.7: 2D ROESY spectra of PMD:β-CD mixture (1:1).

The H10 cis proton of PMD was used to determine the association constants. Graph. 5.8 represents the Scott's plot for the inclusion complex PMD:β-CD.



Graph. 5.8: Typical Scott's plot for PMD:β-CD inclusion complex showing overall association constant (K_a) = 753 M^{-1}.

For the complex PMD:β-CD, an association constant of 753 M^{-1} was calculated. The same work was done with the PMD:HP-β-CD and PMD:γ-CD complexes and the association constants are respectively 283 M^{-1} and 265 M^{-1}. The decrease of K_a is explained by the size of the cavity (too large in the case of γ-CD) as well as the size of the hydroxypropyl groups, which seem to disturb the complexation.

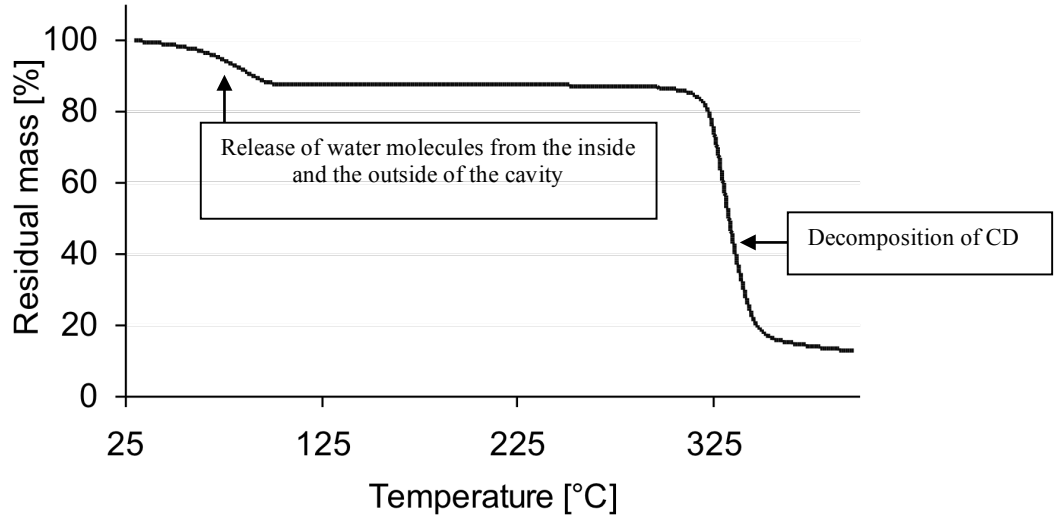
For a repellent product with a long-lasting action, 20 to 25% of active (PMD) is needed. As the PMD will be included in Cyclodextrins, it involves a large quantity of IC dissolved in the product and requires a high solubility in an aqueous medium. Therefore, HP-β-CD and γ-CD will be investigated due to their high solubility in water as shown in Table 5.1.

5.4.2. Preparation and characterization of solid inclusion complex of PMD:CD

Inclusion complexes (IC) can also be prepared under a solid state (powder). PMD complexed as a solid could help its integration into a cosmetic product (cream, lotion etc.) by using aqueous media. Furthermore, HP-β-CD and γ-CD will be studied in details for

the preparation of inclusion complexes with PMD. The characterization of solid IC was performed by ThermoGravimetric Analysis (TGA).

The trend in thermal behaviour for the examined CDs and the values of the thermogravimetric parameters are presented in Graph. 5.9 and Table 5.3. Two thermal effects are seen on this curve. The first slope between 30° and 120°C corresponds to the dehydration of CDs. The second step is related to the decomposition of the CDs' structure.



Graph. 5.9: General TG curve of CDs.

	HP- β-CD		**γ-CD**	
	Temperature range	Mass loss	Temperature range	Mass loss
Release of water molecules	30-92°C	5%	30-158°C	8.8%
Decomposition of CD	298-400°C	88%	325-400°C	70%

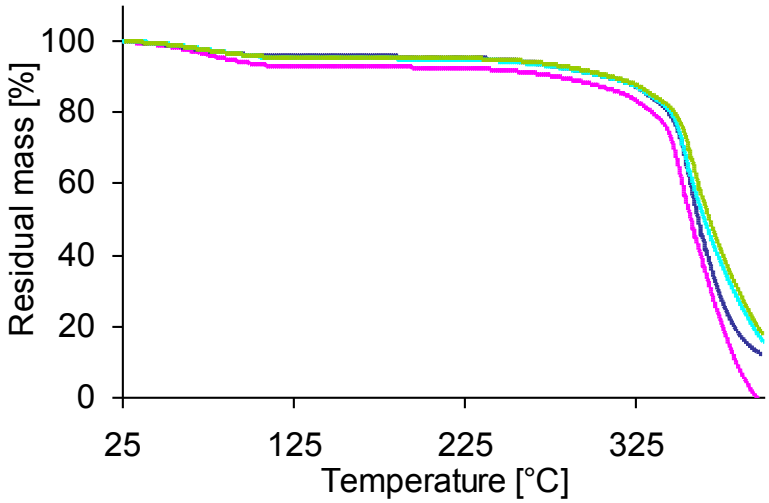
Table 5.3: Thermogravimetric profile of HP-β-CD and γ-CD.

The preparation of solid-state IC was carried out by a simple procedure and the influence of different parameters (time, ratio, presence of alcohol, concentration) was studied. In many cases, the products are a mixture of inclusion complexes, uncomplexed guest molecules and empty hydrated Cyclodextrins. TG measurements enable to distinguish these three forms. When the a complexation occurs, complexes' curves have defined

characteristics. The complexed guest is protected in the CDs cavity and its decomposition is engaged with the CDs decomposition at around 300°C. The complexation of the guest molecules induces less water content in the CDs' by a substitution process. PMD:CD inclusion complexes do not involve the free PMD melting jump at around 200°C as PMD is complexed and not absorbed on the CDs' surfaces.

5.4.3. Evolution of PMD:CD complexation as a function of time

The reaction time was studied in order to determine the minimum time required for the complexation. The percentage of CDs in the medium is fixed at 20 %(w/w) and the mass ratio PMD/CD is 1/10. The kinetic of the complexation was followed over 72 hours. The thermogravimetric profile of the complexation PMD:γ-CD as a function of time is presented in Graph. 5.10.

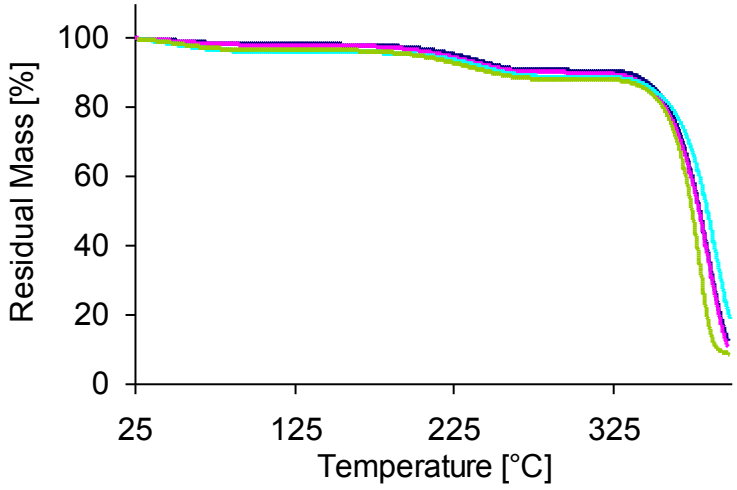


Graph. 5.10: TG curves of PMD:γ-CD complexes after 6h stirring (——), 24h (——) 48h (——) and 72h (——).

The water-loss decreases over the first 24h and no real free-PMD jump is observed on the curves. Therefore, water molecules from the inside of the cavity might be replaced by PMD molecules. Complexation may occur and reach a maximum after 24h. The absorption of PMD mainly occurs when all cavities are occupied by PMD. Also, a small

quantity of absorbed-PMD means that the amount of complexed PMD might be increased.

The same work was carried out using HP-β-CD. The results are presented in Graph. 5.11.



Graph. 5.11: TG curves of PMD:HP-β-CD complexes after 6h stirring ( ), 24h ( ) 48h ( ) and 72h ( ).

The water-loss decreases during the first 6h of stirring and a jump at around 200°C is observed. PMD molecules might have replaced water molecules from the inside of the HP-β-CDs' cavity meaning a complexation of PMD with HP-β-CD. The second slope corresponds to free PMD molecules absorbed on CDs' surfaces. PMD molecules may be absorbed on the hydroxypropyl group of the HP-β-CDs and therefore less PMD molecules are complexed. Table 5.4 gives the percentage of complexed and free PMD in the PMD:HP-β-CD powder.

Reaction Time	Water-loss between 25°C and 120°C (% w/w)	Percentage of replaced water molecules (%w/w)	Free PMD-loss between 120°C and 325°C (%w/w)
HP-β-CD (control)	3.4	-	-
Complexation after 6h	1.6	1.8	7.6
Complexation after 24h	1.8	1,6	8
Complexation after 48h	3.6	-	7.8
Complexation after 72h	3.2	0.2	8.7

Table 5.4: Thermal properties of PMD:HP-β-CD inclusion complexes.

The water-loss decreases during the first 6h and then increases again. Therefore, the maximum of PMD:HP-β-CD inclusion complexes might be reached after 6h. For instance, after a stirring time of 6h, at least 1.8 %(w/w) of the complex contained PMD in the cavity and 7.6 %(w/w) of the complex is PMD absorbed on HP-β-CDs' surfaces.

Some PMD has replaced water molecules from the inside of the HP-β-CDs' cavity but PMD is also absorbed on HP-β-CDs' surfaces. An excess of PMD in the solution will result in the absorption on CDs surfaces. Some further experiments have been carried out using less PMD in order to reduce the amount of absorbed PMD.

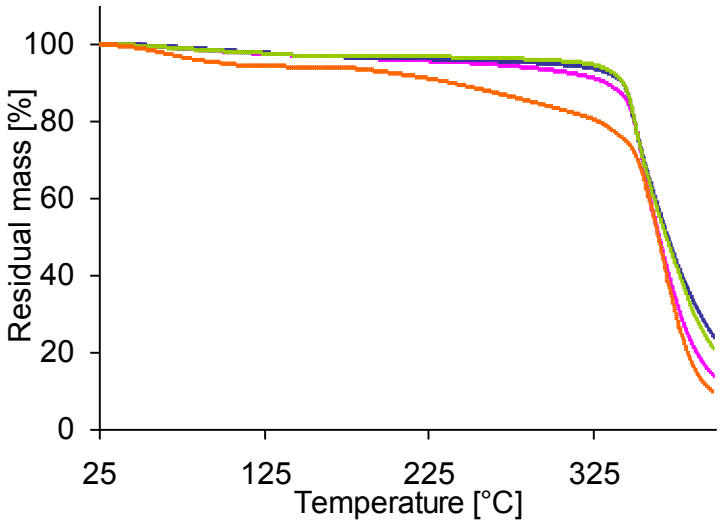
5.4.4. Influence of PMD/CD ratio on the complexation

The ratio PMD/CD was investigated in order to determine the maximum concentration of PMD that can be complexed in CDs cavities. Regarding the previous results, PMD/CD mass ratios from 1/6 to 1/12 have been tested with γ-CD and from 1/8 to 1/20 with HP-β-CD. The conversion mass to molar ratio is described in Table 5.5. These experiments were carried out with 20 %(w/w) of CDs in an aqueous solution at room temperature for 24h and the ratios described in Table 5.6 were used.

Mass ratio PMD/CD	Molar ratio PMD/ γ-CD	Molar ratio PMD/ HP-β-CD
1/6	1/0.8	-
1/8	1/1	1/1
1/10	1/1.33	1/1.23
1/12	1/1.60	1/1.48
1/20	-	1/2.46

Table 5.5: Conversion mass ratio to molar ratio for γ-CD and HP-β-CD.

Graph. 5.12 shows the complex PMD:γ-CD cavity as a function of the ratio PMD/CD.



Graph. 5.12: TG curves of PMD:γ-CD complexes prepared with a mass ratio PMD/CD=1/6 ( ) , 1/8 ( ), 1/10 ( ) and 1/12 ( ).

A PMD/CD ratio between 1/8 and 1/12 does not show a great influence on the TG profile of the PMD:γ-CD complexes. No absorption slope can be observed on the curves. Therefore, PMD:γ-CD complexes are obtained when the amount of PMD increases (PMD/CD= 1/6). The water loss increases and a slope at around 200°C is observed. PMD is in excess and therefore absorbed on γ-CDs' surfaces. Table 5.6 gives the percentage of complexed and absorbed PMD in the powder for different ratio of PMD/CD.

γ-CD:PMD complexation with PMD/CD =	Percentage of complexed PMD in the powder (% w/w)	Percentage of absorbed PMD in the powder (% w/w)
1/6	6.3	10.4
1/8	7.2	5.3
1/10	7.9	2.1
1/12	7.1	1.2

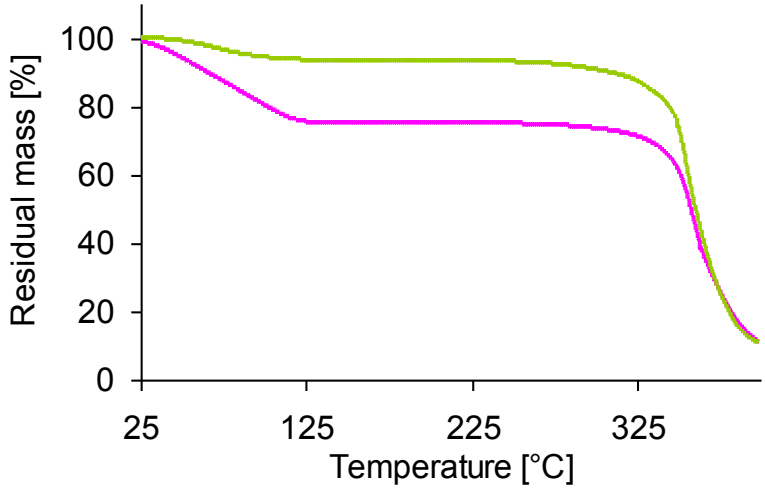
Table 5.6: Percentage of absorbed and complexed PMD with different mass ratio PMD/CD.

While decreasing the amount of PMD in solution, the percentage of complexed PMD in the powder decreases as well as the percentage of absorbed PMD. Nevertheless, the percentage of absorbed PMD is always greater than the percentage of complexed PMD.

With HP-β-CD, complexation might be harder to obtain and absorption of PMD is predominant. A compromise might be reached for a mass ratio PMD/CD of 1/10. The influence of a solvent as well as the concentration of CDs has been then studied in order to improve the percentage of complexed PMD in the powder.

5.4.5. Influence of the addition of Ethanol on PMD:CDs complexation

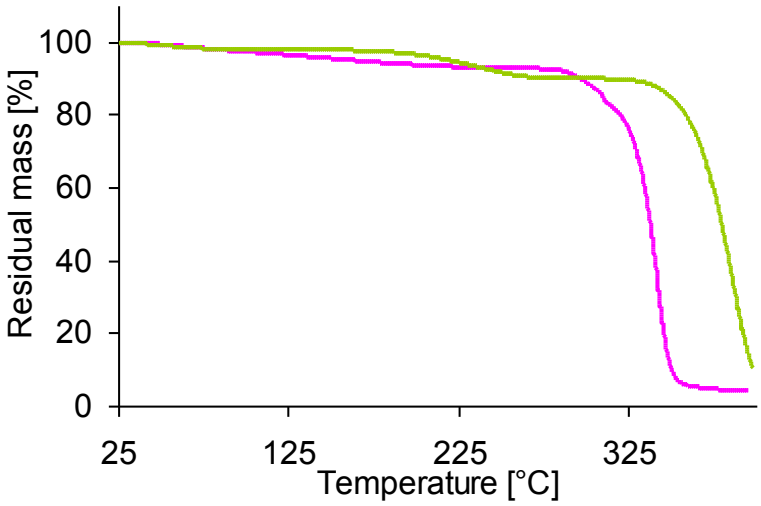
Previous studies have shown the influence of a solvent on complexation[174-176]. PMD is highly soluble in EtOH, therefore the influence of EtOH in solution was investigated. Experiments were carried out 24h with and without EtOH in order to dissolve PMD in water. The percentage of CDs and the PMD/CD mass ratio are fixed respectively to 20% and 1/10. Graph. 5.13 gives the thermal behaviour of the PMD:γ-CD inclusion complexes performed with and without EtOH.



Graph. 5.13: TG curves of PMD:γ-CD complexes prepared with ( ) and without ( ) Ethanol.

No absorption jump is observed meaning that PMD is mainly complexed and less absorbed. The complex prepared with EtOH shows a mass-loss between 25°C and 120°C of 23.4 %(w/w) at 120°C compared to a mass-loss of 6.1%(w/w) for the complex without EtOH. This may be due to the complexation of EtOH into γ-CDs' cavity. The complexation of EtOH reduces PMD complexation in γ-CDs.

The same work was carried out with HP-β-CD and shows different results in Graph. 5.14.

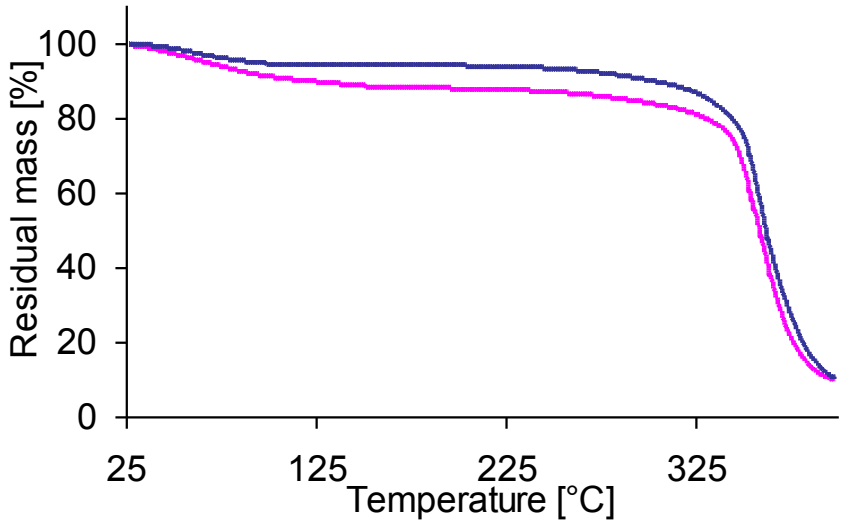


Graph. 5.14: TG curves of PMD:HP-β-CD complexes prepared with ( ) and without ( ) EtOH.

The mass-loss at 120°C does not change but a slight absorption slope is observed. EtOH might complex into the cavities, making them more hydrophobic and therefore allow a better complexation of PMD in HP-β-CDs' cavities. Finally, with EtOH, the complexation of PMD in HP-β-CDs becomes higher than the absorption on the outer surface of CDs.

5.4.6. Role of CDs' concentration on complexation

Regiert and *al* (2002)[172] have demonstrated that the percentage of CDs in solution may influence the preparation of IC. For instance, a high percentage of γ-CD will require a kneading method to stir properly the mixture. In order to use simple methods like co-precipitation or suspension methods[162], a percentage of γ-CD between 0 and 20%(w/w) was used. In the case of the highly soluble HP-β-CD, amounts from 0 to 30%(w/w) were investigated. The complexation occurred at room temperature for 24h and the PMD/CD mass ratio was 1/10. Graph. 5.15 shows the effect of the γ-CDs concentration onto complexation.



Graph. 5.15: TG curves of γ-CD:PMD complexes prepared with 10% (—) and 20% of γ-CD in solution (—).

The absorption of free PMD is low and the water-loss is significantly reduced by using more γ-CDs in solution. While increasing the concentration of γ-CD, the amount of water molecules replaced by PMD molecules increases (from 0 to 4%(w/w)) as well as the percentage of complexed PMD. Therefore, the concentration of γ-CD has to be around 20%(w/w). A higher concentration may induce a better complexation, but requires a kneading method.

In the case of the highly soluble HP-β-CD, Table 5.7 gives the percentage of absorbed and complexed PMD with different CDs' concentrations. PMD molecules are better complexed than absorbed when 20%(w/w) of HP-β-CDs is used.

HP-β-CD:PMD complexation with percentage of CDs in solution	Percentage of complexed PMD in the powder (%w/w)	Percentage of absorbed PMD in the powder (%w/w)
10%	1.1	8.9
20%	7.4	2.6
30%	2.6	7.4

Table 5.7: Percentage of absorbed and complexed PMD with different concentration of HP-β-CDs.

5.4.7. Solubility of PMD:γ-CD and PMD:HP-β-CD inclusion complexes

The solubility of PMD:γ-CD and PMD:HP-β-CD was investigated with samples prepared at room temperature for 24h containing 20% of CDs and a molar ratio PMD/CD of 1/10. For the record, the PMD solubility is 0.29 g/L at 25°C. The PMD:γ-CD complex reached a low solubility (< 0.5% w/w) in water, while the complex PMD:HP-β-CD reached a great solubility to >63.6% w/w. The complex has nearly the same solubility as the pure HP-β-CD (69.6 % w/w).

In conclusion, PMD:HP-β-CD improve PMD solubility in water and could be used to integrate a large quantity of active in a repellent formulation.

5.4.8. Mosquito Repellent Studies

The complexed PMD solution (See Part 5.3.2 for the preparation) was applied on the right forearm and a thick and sticky film was observed on the surface. Same procedure was repeated for the control solution of pure PMD (10% wt) on the left forearm without any relevant effect on the skin.

The control solution provided a mean protection of 110 minutes in the range of previous results observed on *Aedes aegypti* in this bioassay[93]. The protection time is usually extended as a function of the increased concentration of PMD.

The PMD:HP-β-CD complex solution did not provide any repellent protection against *Aedes aegypti*. Mosquitoes were immediately attracted by the human forearm and instantly bitten. Following the EPA guideline (OPPTS 810.3700), the test was stopped at 0 minute protection time.

As seen with the control solution, PMD itself has very good repellent properties on mosquitoes even at low concentrations. The result with the complex is surprisingly disappointing, however a few hypotheses are conceivable. At first, PMD is known to be a contact repellent active, which means that the mosquito needs to be extremely close to the surface or the active to get repelled. By using CDs inclusion complexes, PMD is

encapsulated inside the cavities and is probably not detectable by the mosquitoes. The repellent action on the mosquitoes is therefore limited or negligeable as observed with the previous results. On the other hand, PMD is known to have a low vapour pressure (0.00109 mm Hg at 25°C), which may explain its long-lasting protection. In CDs complexes, PMD may hardly be able to get out of the inner cavity, which may reduce its volatility and therefore decrease the repellent action.

5.5. Conclusion

In this study, the complexation of hydrophobic repellent active *para*-Menthane-3,8-diol with cyclodextrins was considered. ^{1}H NMR spectroscopy and thermogravimetric measurements were used to determine the stoichiometry as well as the association constant of the complexes of PMD with β-CD, HP-β-CD and γ-CD. It was proved that the inclusion complex was formed between PMD and the three CDs. The 1:1 stoichiometry of each complex was determined using the shift variation of CDs and PMD protons caused by the introduction of PMD into the CDs' cavity. The complexation of PMD inside the CDs' cavity was confirmed by 2D ROESY spectroscopy. The preparation of solid PMD: HP-β-CD allowed a higher solubility of PMD complex in water (> 63.6% w/w). Finally, repellent tests with *Aedes aegypti* mosquitoes were carried out on the inclusion complex PMD:HP-β-CD and showed that mosquitoes were not instantaneously repelled. As PMD is a contact repellent, mosquitoes might not reach the active when it is encapsulated in CDs.

PART II

DEVELOPMENT OF A NEW REPELLENT ACTIVE DERIVED FROM *PARA*-MENTHANE-3,8-DIOL

Chapter 6

SYNTHESIS, CHARACTERIZATION OF A NEW ESTER DERIVED FROM *PARA*-MENTHANE-3,8-DIOL SO-CALLED PMD-SUCCINATE AND ANALYSIS OF THE REPELLENT ACTIVITY AGAINST MOSQUITOES

6.1. Abstract

This study involves the esterification of *para*-Menthane-3,8-diol (PMD), a well-known repellent active against mosquitoes, with succinic anhydride. The novel synthesized, hydrophilic compound, sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate so-called PMD-Succinate (PMD-S), was characterized by elemental spectroscopic methods such as IR, ^{1}H-NMR, ^{13}C-NMR, GC-MS respectively. The repellent action of PMD-S was investigated in a bioassay using *Aedes aegypti* mosquitoes on human volunteers and was compared to PMD and *N,N*-diethyl-*m*-methylbenzamide (DEET). The obtained results showed by ANOVA analysis that PMD-S has a high repellent activity statistically comparable to DEET.

6.2. Introduction

Mosquitoes are involved every year in the development of deadly epidemics around the world such as yellow fever, dengue, West Nile virus, chikungunya, Zika[177]. As an example, nearly half of the world population is at risk of malaria and 220 million cases have been reported in 2015[125,178,179]. The control of these epidemics depends on the prevention and therefore the ability to reduce the risk of contamination[180,181]. The use of mosquito skin repellents is one of the most effective ways for a personal protection. Repellent actives like *N,N*-diethyl-*m*-methylbenzamide (DEET), ethyl 3-[(acetyl)(butyl)amino]propanoate (IR3535), 1-piperidincarboxylid acid 2-(2-hydroxyethyl)-1-methylpropylester (KBR3023), *para*-Menthane-3,8-diol (PMD)[2,182,183] are often used in formulations to provide a long and efficient protection for human beings against blood-sucking arthropods. The major drawback of these actives while integrating into formulations, is their poor solubility into aqueous mediums. Therefore, formulators often use organic solvents (^{i}PrOH, EtOH) to render their actives soluble. For instance, EtOH, which is often integrated into repellent formulations as a solvent or co-solvent, has been widely reported as an efficient skin penetration enhancer in the concentration of 5–100%[184,185] and thus decreases the lasting protection of the product. However, the formulators try to avoid the use of alcohol and replace this solvent by a water medium and the integration of more surfactants. The use of surfactants may increase the toxicity on a skin compatibility aspect; allergenic reactions are often claimed while using high concentrations[186-189]. Also, the consumer market is more and more concerned on the risks related to the use of high concentration of PolyEthyleneGlycol (PEG) derivatives, Ethoxylated fatty acids, Ethoxylated fatty alcohols (EO) derivatives (presence of ethylene oxide, dioxane, polycyclic aromatic compounds and heavy metals), mainly employed in cosmetic products[190,191]. Current initiatives focus on widely replacing chemical surfactants with bio-derived or natural alternatives in the long term[192-194].

Nowadays, *para*-Menthane-3,8-diol is often used for its high repellent activity against different species of mosquitoes, as well as for its low toxicity. PMD is a mixture of cis and trans isomers with a 62/38 ratio. The trans isomer exhibits better repellency, whereas cis provides a longer repellent activity[70]. However, the major drawback of PMD is a low solubility in water (0.29g/L at 25°C).

The present study relates to a fast and easy mono-esterification of *para*-Menthane-3,8-diol with the anhydride of succinic acid to provide a new PMD ester with a high solubility in aqueous mediums. The succinic anhydride can be obtained from succinic acid by fermentation of vegetarian raw materials[195-197] and PMD from a natural source (See Chapter 2). Furthermore, the repellent activity on mosquitoes of the synthesized compound was tested in a bioassay with *Aedes aegypti* mosquitoes.

6.3. Experimental procedures

6.3.1. Chemical

Oxolane-2,5-dione (succinic anhydride: Sigma Aldrich, France), sodium methanolate (sodium methoxide: Sigma Aldrich, France), 5-methyl-2-propan-2-yl-cyclohexan-1-ol (menthol: Takasago, France), 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Takasago, France), *N,N*-diethyl-*m*-methylbenzamide / DEET; Sigma Aldrich, Germany), were used as received. Methylbenzene (toluene), methanol, ethanol, propan-2-ol (isopropanol), pentane, disodium hydrogenphosphate dihydrate (Na_2HPO_4) and sodium dihydrogenphosphate monohydrate (NaH_2PO_4) were obtained from Merck (Germany).

6.3.2. Analytical equipment

Gas phase chromatography (CPG 6890N(G1530N), agilent) GC analysis were performed on a HP 6890 Series gas chromatograph fitted with a flame ionization detector (FID) and an electronic integrator. The capillary column used was a HP model 19091 J-413: HP-5 5% Phenyl Methyl Siloxane (30 m x 320 μm; film thickness 0.25 μm nominal). Helium was employed as a carrier gas at a flow rate of 104 ml/min and 65.6 kPa inlet pressure. The temperature program was: 60°C at 10°C/min, rising to 180°C, and remaining at 180° for a period of 5 min, then rising at 10°C/min to 280°C, and remaining for a period of 5 min. Injector and detector were maintained at 300°C.

NMR spectroscopy ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker AC 300 instrument with D_2O or $CDCL_3$. The chemical shifts were expressed as a δ unit in parts per million (ppm). The following abbreviation is used: s = singlet, d = doublet, t = triplet; q = quartet; m = multiple; br. = broad.

Infrared spectroscopy The Infrared spectra were recorded on a Thermo Scientific Nicolet 6700 FT-IR diamond 30.000-200 cm^{-1} spectrometer. IR bands were expressed as cm^{-1}.

Mass spectrometry Mass spectra were recorded on a Waters Xevo-QT using electrospray ionization.

6.3.3. Synthesis of PMD-S

First step: Succinic anhydride (5.82g, 0.058 mole) was added to PMD (10g, 0.058 mole) in toluene (35 mL). The reaction mixture was stirred and heated under reflux for 20 hours. The reaction was monitored by GC. Toluene was removed under vacuum and the precipitate was recrystallized from methanol and filtrated.

Second step: PMD succinate acid (14.7g, 0.054 mole) was dissolved in MeOH (140 mL). CH_3ONa (5.4g, 0.1mole) were dissolved in MeOH (200 mL) and added drop-wise to the previous solution. MeOH was removed under vacuum and the precipitate was recrystallized with a pentane/diethyl ether (2/1) solution and filtrated.

Sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate : as viscous colorless oil, yield 39.72%; **IR** ($\nu \cdot cm^{-1}$): 2925 (ν OH); 1710 (ν C=O); 1157 (ν C-O); **^{1}H-NMR** (δppm): 0.79 (d, *J* =6.6Hz, 3H, CH_3 ; *H4 cis*), 0.81 (d, *J* =6.5 Hz, 3H, CH_3 ; *H4 trans*), 0.84-1.0 (m, 2H, CH_2 ; *H5,H5'*), [(0.9-1.0)-(1.90-1.94)] (m, 2H, CH_2 ; *H2,H2'*), 1.07 (s, 3H, CH_3 ; *H9-10*), 1.10 (s, 6H, CH_3 ; *H9-10*), 1.14 (s, 3H, CH_3 ; *H9-10*), 1.27-1.33 (m, 1H ; *H7*), 1.4-1.5 (m, 1H, CH, *H3* trans), 1.55-1.64 (m, 1H, CH ; *H3 cis*), 2.48-2.80 (m, 8H, CH_2 ; *H11-12*), 4.8 (m, 1H, CH ; *H1* trans), 5.34 (m, 1H, CH ; *H1* cis); **^{13}C** (δppm): 21.7 (CH_3 ; C4 trans), 22 (CH_3 ; C4 cis), 22 (CH_2 ; C6 cis), 25 (CH_3 ; C9-10 trans), 26.6 (CH ; C3 cis), 27.2 (CH2), 27.7 (CH_3 CH_3 ; C9-10 cis), 28.5 (CH_3 ;

C10-9 cis), 28.5 (CH_3 ; C10-9 trans), 28.8 (CH_2 ; C12 cis/trans), 29.7 (CH2 ; C11 cis/trans), 31.2 (CH ; C7 trans), 34.2 (CH_2 ; C5 trans), 34.7 (CH_2 ; C5 cis), 39.4 (CH_2 ; C2 cis), 40.8 (CH_2 ; C2 trans), 49.6 (CH ; C7 cis), 51.4 (CH ; C3 trans), 71.7 (CH ; C1 cis), 72.4 (Cq ; C8 cis), 73.5 (CH ; C8 trans), 76.3 (CH ; C1 trans), 171.5 (Cq ; C14 trans), 171.9 (Cq ; C14 cis), 176.1 (Cq ; C13 cis), 176.3 (Cq ; C13 trans); **MS:** ES(-) m/z : [M-H+] 271.1542 (37%) ; 116.9300 (48%) ; 99.0073 (100%).

6.3.4. Mosquito repellent studies

Insects Strain of *Aedes aegypti* mosquitoes from Bayer AG were reared according to the standard protocol at 27°C, a relative humidity of 60–80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in H2O filled with a 1:1 mixture of tap- and deionised H_2O, and fed with fishfood flakes (Tetra Min®). Before hatching, the pupae were transferred to a cage (40 x 30 x 20 cm) and provided with sugar soln. (10% dextrose). Mosquitoes at an age of 9-14 days were used for the tests.

Volunteers Five human volunteers aged between 20 and 26 participated in the mosquito cage test bioassay. No abnormal allergic reaction after application of the formulations was observed.

Laboratory tests Human skin tests were conducted as described below.
Application of repellents: The skin of the forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% iPrOH and then dried with a paper towel. An area larger than the test window was marked on the skin with a metal template. According to the United States EPA, 1 g of pump spray is applied per 600 cm² skin. The marked area had a size of 98 cm²; therefore 0.2 g were applied to the test area. The test substance was applied using a pipette with disposable tips, for each test person a new tip was used. The test substance was spread evenly on the skin of the forearm wearing a latex glove.
Exposure to mosquitoes: Thirty mosquitoes were placed in a test cage that was fitted with a test window in the floor of the cage. The test window was closed by a metal slide and was opened by inserting a metal frame for the exposure of the treated quadratic (98 cm²)

skin area (ventral) and untreated area (back) of the volunteer´s forearms. This method was designed so that each volunteer served as his own control.

Zero control: Zero control is the untreated back of the forearm of the test person. A window frame with mosquito net is used to keep probing mosquito from successfully taking a blood meal. Biting pressure must exceed 10 probings in 30 s. After 30 s and more than 10 probings the mosquitoes were considered as active and suitable for the experiment.

Test proper: Each test person was assigned to one test cage. Between two tests a special air ventilation system was attached to the cage in order to prevent any accumulation of odours and active substances in the cage. The treated skin was exposed to the mosquitoes for a testing time of 2 min. In this time, the number of landings and bitings on the treated skin was noted and compared to the untreated control skin.

Determination of duration of protection: As far as there is no official protocol in EU, tests were based on the EPA draft guideline OPPTS 810.3700 (EPA, 2000). The criteria to define a complete protection time were dependent on the zero control. A 15 minutes margin of error was attributed on every result.

6.3.5. Statistical analysis

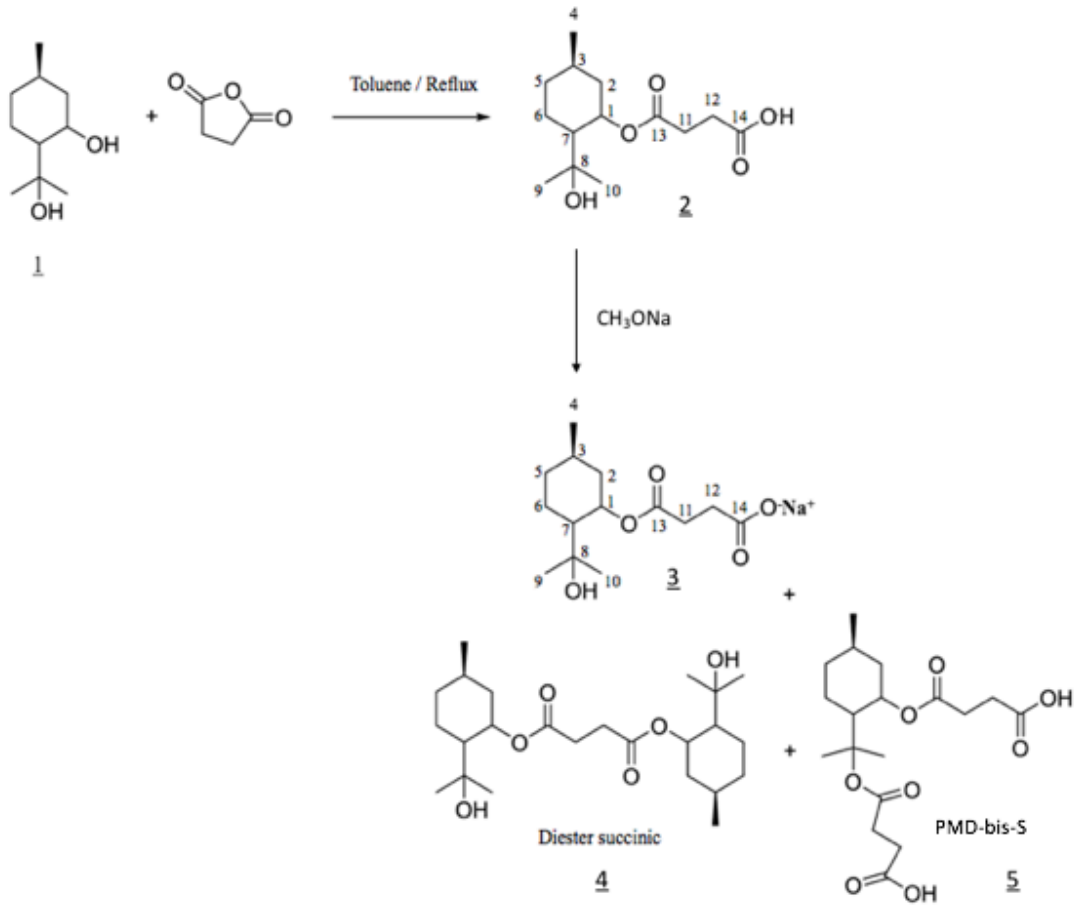
Data reported as protection time were subjected to an analysis of variance (ANOVA) and Post-Hoc T-test ($P<0.05$) using the software IBM SPSS Statistics (version 22 for windows). This statistical analysis was used to check the reliability of the results obtained from the assay.

6.4. Results and discussion

6.4.1. Synthesis

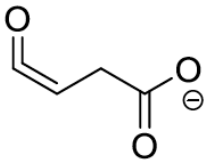
The synthesis of *para*-Menthane-succinate (PMD-S) (**3**) consisted of the esterification between *para*-Menthane-3,8-diol (PMD) (**1**) and succinic anhydride (equimolar concentration) in refluxing toluene during 8 hours to afford PMD-Succinate acid (**2**) as shown in Scheme 6.1. The second step involved an acid-base reaction between PMD-

Succinate acid and sodium methoxide in methanol, thus forming the hydrophilic head of the new repellent active. The synthesis was monitored by GC.



Scheme 6.1: Synthesis of *para*-Menthane succinate (PMD-S (3).

PMD-S (**3**) was characterized by elemental analysis IR, ^{1}H and ^{13}C NMR as well as by MS. The IR (cm^{-1}) showed the characteristic signal of C=O at 1710 cm^{-1}, a vibration band of C-O at 1157 cm^{-1} and an elongation vibration of OH at 2925 cm^{-1}. High-resolution mass spectrometry confirmed the structure of **(3)** with the pseudo-molecular ion $[M\text{-}H^{+}]$ observed in negative ESI at *m/z* = 271.15 corresponding to the molecular weight of the molecular formula $C_{14}H_{22}O_5$. The characteristic ions at *m/z* = 116.93 and m/z = 99.007 corresponding to the fragment shown in Scheme 6.2 are observed.

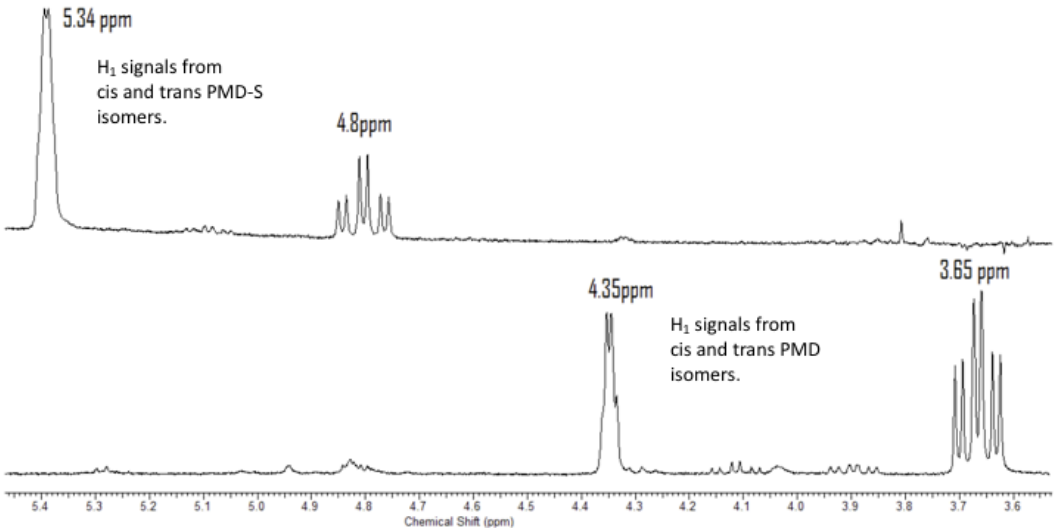


Scheme 6.2: PMD-S characteristic fragment at *m/z* = 99.00.

The mass spectra also gave the molecular ion [M^+] at m/z = 371.17, which may be assigned to the structure of the impurity PMD-bis-Succinate (**5**) with the molecular weight of the formula $C_{18}H_{27}O_8$. Compound **(4)** was not observed by MS.

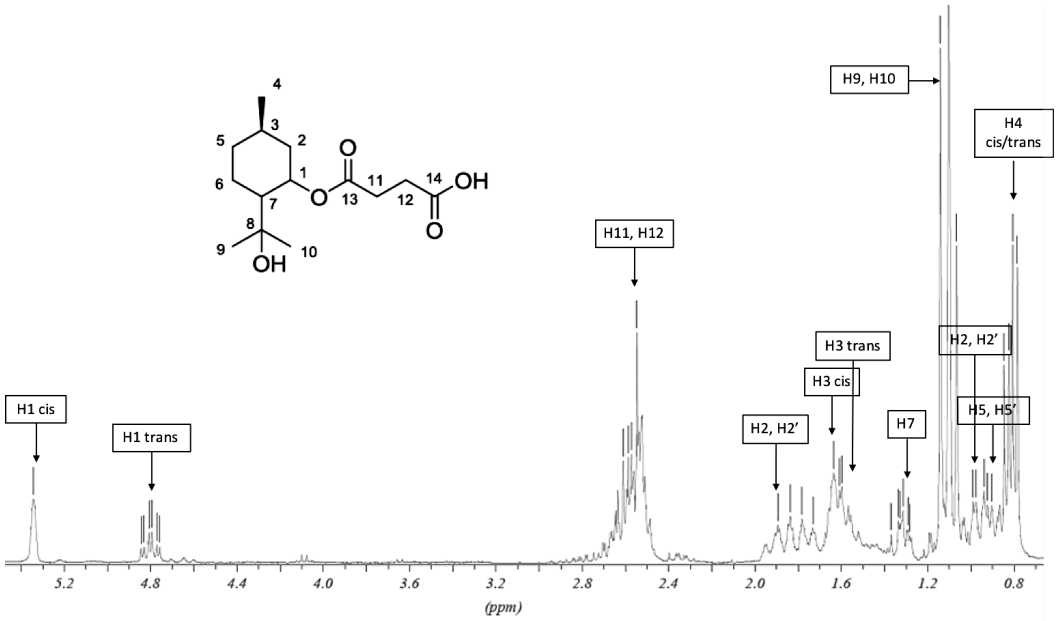
The ^{1}H NMR spectra (see Graph. 6.2) revealed the characteristic signals of PMD-S. The two CH_2 from the succinic chain ($H_{11\text{-}12}$) were observed at 2.4-2.8 ppm. The CH_3 (s) of the C_9 and C_{10} are slightly deshielded in comparison with the same CH_3 of the PMD. The CH_3 (d) from the C_4 was also deshielded.

The H_1 signals allowed us to distinguish the PMD-S from the PMD (Graph. 6.1). As for the PMD, two signals were obtained for the two cis and trans isomers: cis isomer at 5.4 ppm (br. m, poorly resolved), trans isomer at 4.8 ppm (td) with J_1: 4.5Hz and J_2: 10.5Hz. These two signals are strongly deshielded in comparison with the PMD (about 1 ppm), which corresponds to the attractive inductive effect of the ester function.

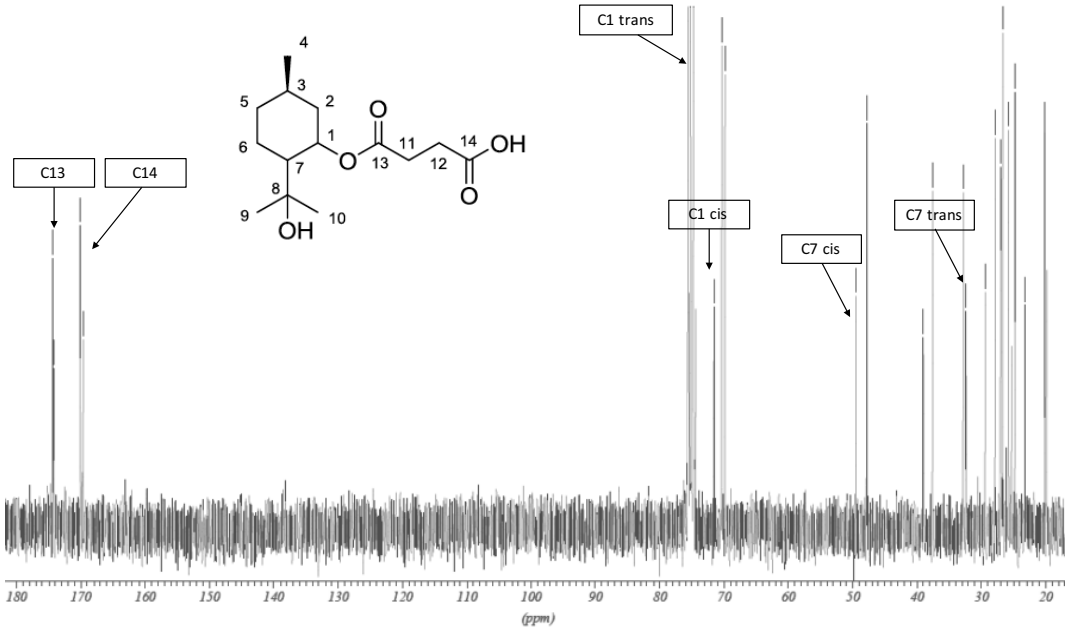


Graph. 6.1: ^{1}H NMR ($CDCL_3$-300MHz) spectra from PMD and PMD-S.

The ^{13}C NMR spectra (See Graph. 6.3) revealed the carbonyl signals at 171-176 ppm and a deshielding effect of the C_1 carrying the ester function at 71.7 and 76.3 ppm for the cis and trans isomers respectively.



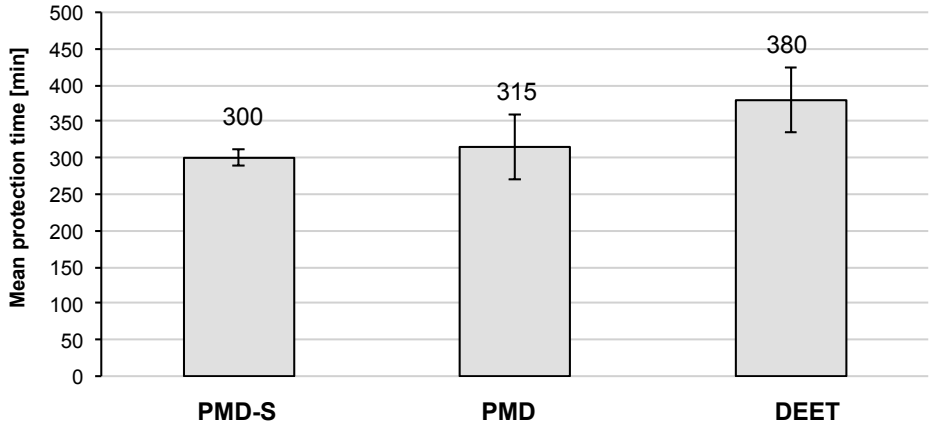
Graph. 6.2: ^{1}H NMR spectra of PMD-Succinate.



Graph. 6.3: ^{13}C NMR spectra of PMD-Succinate.

6.4.2. Study of repellent properties

PMD-Succinate obtained in this study was subjected to repellent tests in a bioassay using *Aedes aegypti* mosquitoes and human volunteers as described in part 6.3.3. *N,N*-diethyl-*m*-methylbenzamide (DEET) and *para*-Menthane-3,8-diol (PMD) were used as controls and were prepared by dissolving 20% weighed amount of repellents in ethanol whereas PMD-S was solubilized in Millipore water. The 20% solutions were applied on the right forearm showing no specific irritation on the skin. Results of the bioassay tests are shown in Graph. 6.4.



Graph. 6.4: Duration of repellent activity on *Aedes aegypti* with PMD-S, PMD, and DEET.

According to the results, it was determined that PMD-S generates a repellent activity with a mean protection time of 300 minutes on human volunteers in comparison to 315 min with PMD and 380 min with DEET. The time protection with the two controls repellents is comparable to previous studies (See Chapter 2,3).

One-way ANOVA showed no significant repellent difference between the three actives ($P > 0.05$; see Table 6.1)

Repellent	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
PMD-S	**300**	**26.22**	**11.73**	**267.44**	**332.56**	**265**	**330**
PMD	315	82.01	36.67	213.18	416.82	210	420
DEET	380	100.31	44.86	255.45	504.55	270	525

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	18083.333	2	9041.667	1.552	**0.251**
Within Groups	69900.000	12	5825.000		
Total	87983.333	14			

Table 6.1: Time protection of the three repellent samples PMD-S, PMD and DEET. The difference is significant at $p<0.05$.

PMD-Succinate should hydrolyse and release PMD molecules over time. Considering the molecular weight of PMD-S (294.34 g/mol) and PMD (172.26 g/mol), the new ester contains only 58.5% of the PMD base. A 20% aqueous solution of PMD-S should release around 11.7% PMD by its hydrolysis process. According to previous studies (see Chapter 3), 12% PMD solution using the same bioassay provided a mean protection time of 150 min. If there were a release of this ester from its hydrolysis (pH or enzymatic hydrolysis), the protection time of PMD-S would have been close to the 12% PMD solution, which was not the case in this study. The non-significant difference between the three tested solutions showed noticeably that the newly ester acted as a repellent active on its own with an action close to the standard reference DEET.

6.5. Conclusion

In this study, we reported the synthesis of a novel hydrosoluble mosquito repellent active sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate so called PMD-Succinate (PMD-S), synthesized from *para*-Menthane-3,8-diol and succinic anhydride (yield 39.72%). The structure of the newly synthesized compound was confirmed by elemental analysis: IR, ^{1}H-NMR, ^{13}C-NMR spectra and GC-MS. Additionally, repellent studies on *Aedes aegypti* mosquitoes were investigated on the new molecule and showed a high repellent action (300 min) statistically comparable to the standard active DEET. Using a soluble repellent active without the addition of any surfactants or solubilizers for skin leave-on formulations may be a great benefit for any industrials aching to reduce the toxicity or the price of a repellent product.

Chapter 7

PHYSICAL AND CHEMICAL PROPERTIES OF THE REPELLENT ACTIVE *PARA*-MENTHANE-3,8-DIOL (PMD-S)

7.1. Abstract

Following the work in Chapter 6 on the synthesis of the novel repellent active PMD-Succinate, investigations on physical and chemical properties were reported. The Krafft point and pKa were measured. As a result, the ester acts as a hydrotrope with a good surface activity and a Minimum Hydrotrope Concentration (MHC) of the order of 0.39M similar to other hydrotropes. Furthermore, a binary phase diagram has been established showing ellipsoidal open-layer assemblies with low viscosity, and a cubic phase at high concentrations. The hydrolysis of PMD-S was also studied showing high stability both in basic and in acidic mediums. Due to its high solubility in water (>250g/L at 25°C) and its hydrotropic function, a ternary phase diagram was studied with the system PMD-S/PMD/Water in order to design long-lasting and alcohol-free repellent formulations suitable for the market.

7.2. Introduction

Active ingredients such as *N,N*-diethyl-*m*-methylbenzamide (DEET), ethyl 3-[(acetyl)(butyl)amino]propanoate (IR3535), 1-piperidincarboxylid acid 2-(2-

hydroxyethyl)-1-methylpropylester (KBR 3023), *para*-Menthane-3,8-diol (PMD) are often used in product formulations to ensure effective long-lasting protection against blood sucking arthropods[100,101,198]. A considerable disadvantage of these actives is their low solubility in water. Organic solvents, such as for instance ethanol or isopropanol, and/or surfactants are used in the formulation system to render soluble the active in an aqueous phase[138]. However, it is well-known that ethanol may significantly increase and accelerate the penetration of active ingredients into the upper skin layers[137,199]. This result also leads to skin irritations and allergic reactions as a function of the used solvent[200]. For this reason, in modern formulations alcohols are partly replaced by surfactants. However, the use of surfactants also was disadvantages in the form of possible skin damages[201-203]. In Chapter 6, a study has set the task of developing a water-soluble repellent active derived from PMD called PMD-Succinate (PMD-S), in the formulation of which no undesirable additives are used and which keeps an activity and a duration comparable with those of current insect repellents actives.

Throughout the present paper, the general properties of PMD-S are studied especially to check if the new PMD ester acts either as a surfactant or a hydrotrope. Hydrotropes are a particular class of compounds that exhibit distinct solution properties[204,205]. They may self-associate in aqueous medium, comparable to amphiphile self-association but without showing micellization[206-208]. They are efficient solubilizers and can influence the formation of micelles and microemulsions as well as viscosity. The classification of hydrotropes on the basis of molecular structure is difficult, since a wide variety of compounds has been reported to exhibit hydrotropic behaviour[209,210]. The term minimum hydrotrope concentration (MHC) has been used in consonance with the CMC of surfactants[211,212]. MHC corresponds to the hydrotrope concentration at which the increase in the hydrophobic compound solubility becomes significant. Cooperative aggregation such as self-association is accompanied by such a phenomenon[210,213]. Considering its short hydrophobic tail and its big polar head group, PMD-Succinate would belong more to a hydrotrope group than a surfactant one. Krafft point, pKa, surface activity, solubilisation, binary phase diagram will be measured in order to characterize the physical and chemical properties of PMD-Succinate.

7.3. Experimental procedures

7.3.1. Chemical

Oxolane-2,5-dione (succinic anhydride: Sigma Aldrich, France), sodium methanolate (sodium methoxide: Sigma Aldrich, France), 5-methyl-2-propan-2-yl-cyclohexan-1-ol (menthol: Takasago, France), 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Takasago, France). Methylbenzene (toluene), methanol, ethanol, propan-2-ol (isopropanol), pentane, ethoxyethane (diethyl ether), 2-[4-(2-Chloro-4-nitrophenylazo)-N-ethylphenylamino]ethanol (DR-13), disodium hydrogenphosphate dihydrate (Na_2HPO_4) and sodium dihydrogenphosphate monohydrate (NaH_2PO_4) were obtained from Merck (Germany). 2-Hydroxypropanoic acid (lactic acid) was purchased from Purac (Netherlands).

7.3.2. Analytical equipment

Gas phase chromatography (CPG 6890N(G1530N), Agilent) GC analysis were performed on a HP 6890 Series gas chromatograph fitted with a flame ionization detector (FID) and an electronic integrator. The capillary column used was a HP model 19091 J-413: HP-5 5% Phenyl Methyl Siloxane (30 m x 320 µm; film thickness 0.25 µm nominal). Helium was employed as a carrier gas at a flow rate of 104 ml/min and 65.6 kPa inlet pressure. Temperature program was: 60°C at 10°C/min, rising to 180°C, and remaining at 180° for a period of 5 min, then rising at10°/min to 280°C, and remaining for a period of 5min. Injector and detector were maintained at 300°C.

Surface activity Surface tensions were measured at 25°C at different concentrations of monoester using a Processor Tensiometer K100 (Krüss, Germany). All measurements were made as a function of time until stabilisation of the surface tension.

Determination of the Krafft point The Krafft point was determined visually on aqueous solutions containing 1% (w/w) of the monoester. After dissolving the active, the solution was left one day at 4°C in order to precipitate the active ingredient. Then, it was

progressively heated and the onset of solubility was taken as the Krafft point. pH was fixed at 10 to obtain clear solution at the beginning.

Polarized light optical microscopy At first, the lyotropic phase behaviour was investigated by polarizing optical microscopy using the isothermal concentration gradient method. A small amount of powder was placed on a slide with a free cover slip; water was allowed to penetrate by capillarity. Birefringent phases were then observed at different temperatures with an Orthoplan polarizing optical microscope supplied by Leitz (Germany). Then samples of given composition were prepared by introducing weighted amounts of the active ingredient and water into a tube. The samples were homogenized and viewed with the microscope as a function of temperature.

Hydrolysis of the monoester PMD-S was introduced into 5 mL of aqueous solutions at 0.25M at various pH values buffered at pH 5 (NaH_2PO_4 0,05M/Na_2HPO_4 0,05M), pH 7.0 (NaH_2PO_4 0.1 M/NaOH 0.058 M) and pH 11.0 (Na_2HPO_4 0.05 M/NaOH 0.054 M)). The pH was kept constant by additions of NaOH (1 M) and/or lactic acid (1 M). Hydrolysis of the surfactant was followed by GC.

Absorbance experiments All experiments concerning the solubilization process and the optical density measurements were done in a thermostated room at 25 ± 0.2°C. All solutions to be measured, i.e. containing water and the studied compound at an appropriate concentration, were saturated with a sufficient amount of dye Disperse Red 13 (DR-13) and left equilibrated 24h. The solutions were then filtered in order to separate the non-solubilized excess of dye from the solutions. The optical density (OD) of the filtered solutions were measured in 1 cm path length quartz cells with a UV-visible spectrophotometer Cary-3E at a wavelength of 525 nm corresponding to the wavelength λ_{max} of the DR-13 where the dye has its absorption maximum. Before each measure a zero absorbance was done with the corresponding solution without dye. For the studied hydrotrope, λ_{max} values were found to be not so sensitive to the chemical nature of these compounds. When the measured OD was above a critical value, suitable dilutions were done by using the same solution but without dye. The resulting ODs reflect then, through the Beer-Lambert law, the concentration of the hydrophobic dye solubilized in the corresponding aqueous hydrotrope solutions mixtures.

Dynamic Light Scattering (DLS) measurements Droplet size analysis was performed using a commercial goniometer (CGS-2, ALV-GmbH Langen, Germany) equipped with a vertical-polarized 22 mW HeNe laser (wavelength = 632.8 nm), a fiber optical detection unit with an avalanche photodiode, and an ALV-5000/E multiple correlator. The scattering angle was 90° and the intensity autocorrelation functions were analysed using the software ALV-5000/E-Win V1.4.8 (ALV-GmbH, Germany). All measurements were performed at 25°C.

Rheology measurements All rheological properties were measured with a Rheometer CVO 120 apparatus (Bohlin instruments, England). A cone-plate sensor was used with a diameter of 40 mm and cone angle of 4°.

Thermo-gravimetric analysis Thermo-gravimetric measurements were performed under nitrogen flow. A TGA 7 from Perkin Elmer corp. (USA) was used. A heating program was settled up at 33°C during 24h. A constant gas flow of 25ml.min^{-1} was set for all the tests. The precision of temperature measurement for the thermobalance is ±1°C. The residual mass was plotted against the time.

7.3.3. Synthesis of PMD-S

13.1g (0.13 mole) of succinic anhydride, 22.5g (0.13 mole) of PMD and 66 ml of toluene were placed in a round-bottomed flask. The mixture was stirred in an argon atmosphere for 8 hours and heated under reflux, while the reaction course was monitored by GC. Toluene was then removed under reduced pressure and the residue recrystallized from methanol.

24.24g (0.1 mole) were dissolved in 140 ml of methanol and place in a round bottomed flask. 5.4g (0.1 mole) of sodium methoxide were dissolved in 200 ml of methanol. The solution was added dropwise under stirring with the PMD-S solution and cooled with an ice bath. After complete addition, the methanol was removed under reduced pressure and the residue recrystallized from pentane/diethyl ester (2/1).

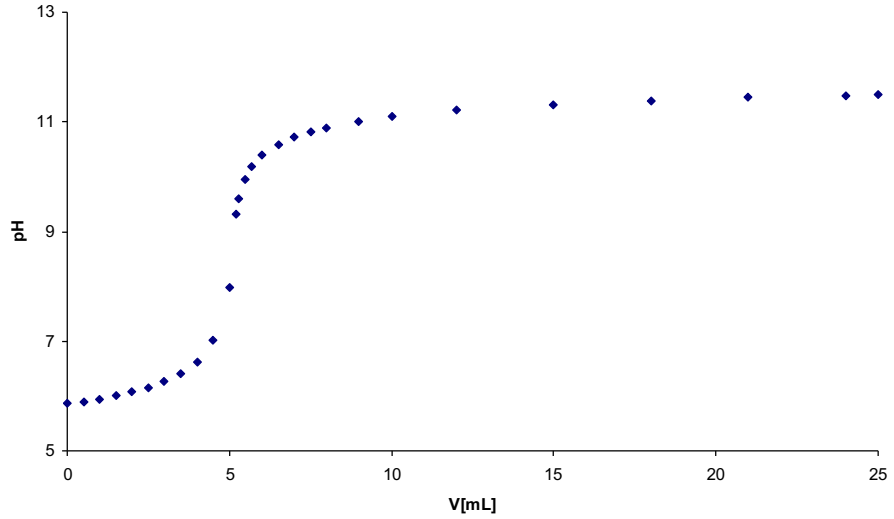
7.4. Results and discussion

Determination of the solubilisation temperature

The Krafft point of PMD-Succinate is below 0°C. The introduction of an ester function in the alkyl chain has a significant effect on the Krafft point. This behaviour may be explained by the polarity of the ester functions thus providing a higher hydrophilicity. They break the regularity of the alkyl chain, by making the ester more easily dissolved. Moreover, the OH group on the hydrophobic chain may improve the hydrophilicity of the molecule. Nevertheless, the determination of this Krafft point is not a determining factor to conclude if PMD-S is a surfactant or a hydrotrope.

Determination of pKa

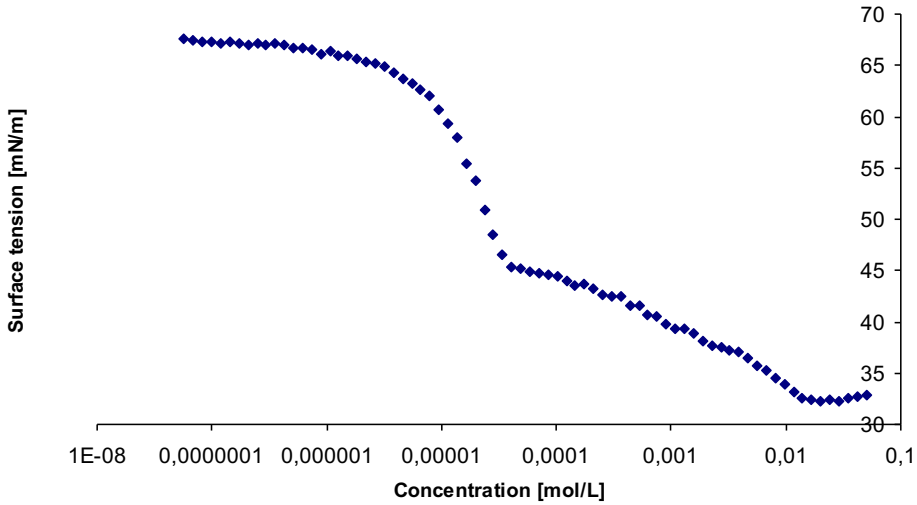
The pKa of PMD-S is around 6.1 as shown in Graph. 7.1 while typical pKa of carboxylates are of the order of 4-5. This phenomenon is due to the self-aggregation of PMD-S molecules. Nevertheless, it is not yet possible to determine if such aggregates are micelles or only self-assembly molecules. The presence of impurities in the medium (for example PMD-bis-Succinate), even in small proportions, may also disturb the pKa. Additionally, the presence of an insolubility area between 0.1% and 9% (w/w) may be due to the spontaneous protonation of the carboxylate anion and the formation of the PMD-Sucinnate acid[214,215].



Graph. 7.1: Determination of PMD-Succinate pKa.

Determination of surface activity

The evolution of the surface tension as a function of the PMD-Succinate concentration can be observed in Graph. 7.2. PMD-S shows a decrease of the surface tension of 30mN/m. This result is comparable to other anionic surfactants and some hydrotropes[216-218] as shown in Table 7.1.



Graph. 7.2: Evolution of the surface tension as a function of PMD-Succinate concentration.

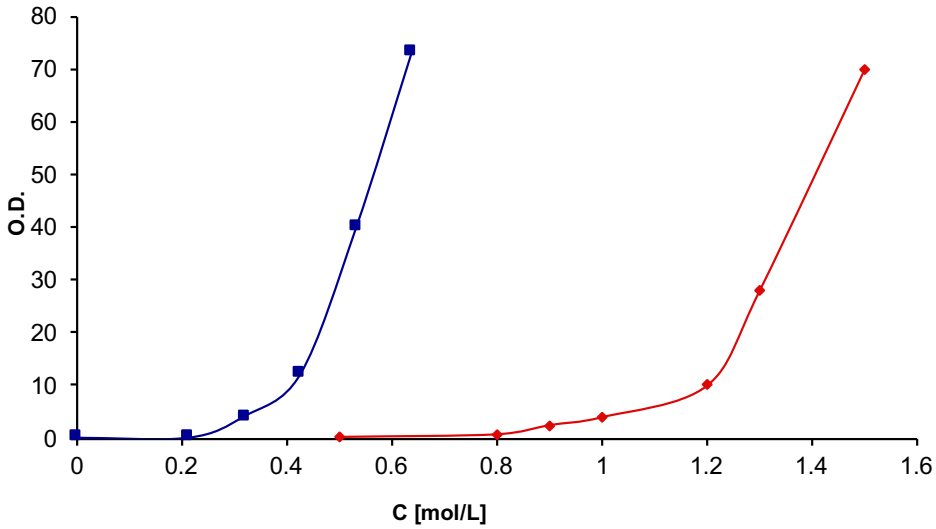
Hydrotrope	Surface activity
PMD-S	72 mN/m to 32 mN/m
Sodium Xylene Sulfonate	72 mN/m to 53 mN/m
Sodium Cumene Sulfonate	72 mN/m to 46 mN/m

Table 7.1: Surface activity comparison of PMD-Succinate with the two hydrotropes SXS and SCS.

The concentration-dependent reduction in the surface tension is more gradual with PMD-S in comparison to the sharper drops encountered with micellar surfactants. Moreover, the break of the curve cannot be considered as the CMC. The measured Minimum Aggregation Concentration is around 1.4 10^{-2} mol/L, which is too low for such a molecule. In fact, PMD-S shows a big polar head and a short hydrophobic tail that would provide a higher value at around 10^{-1}M or more. Nevertheless, the data in Graph. 7.2 suggest that PMD-Succinate self-aggregates beyond a given concentration in water.

Determination of Minimum Hydrotrope Concentration (MHC)

In order to establish the nature of PMD-S, the solubilization of the dye Disperse Red 13 (DR-13) has been studied[210]. As shown in Graph. 7.3, the curve of the PMD-S shows a hydrotrope behaviour.



Graph. 7.3: Evolution of the Optical density (OD), related to the amount of dissolved DR-13 dye as a function of the molar concentrations of PMD-S in water (—■—) and Sodium Xylene Sulfonate in water (—♦—).

The MHC of PMD-S is around 0.39M. This result is close to the MHC of others hydrotropes as shown in Table 7.2. However, the slope of the curve demonstrates a high solubilization capacity. A quantitative comparison of the hydrotropic behaviours can be done by comparing the slopes[210]. The higher the slope is more the solubilization capacity is important. As shown in Graph. 7.3, the slope of PMD-S is higher than the slope of SXS, which is already considered as an efficient hydrotrope.

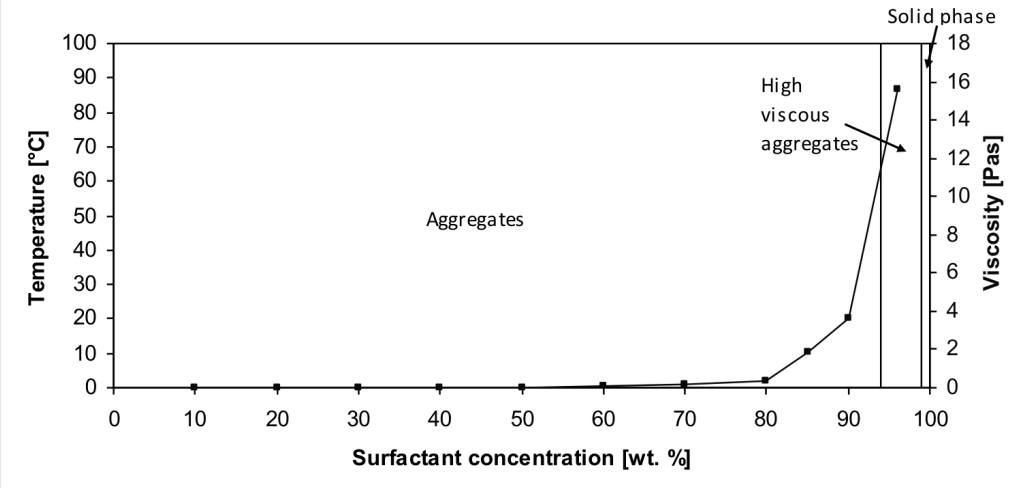
Hydrotrope	MHC
PMD-S	0.386 M
Sodium Xylene Sulfonate	0.6 M
Sodium Cumene Sulfonate	0.1 M

Table 7.2: Minimum Hydrotrope Concentration of PMD-S in comparison to SXS and SCS.

Binary phase diagram

The knowledge of the liquid crystal behaviour is a key to understand the surface properties of a molecule and therefore, binary phase diagrams are investigated. The general pattern of liquid crystals with mono-alkyl surfactants is well established[219]. Single-charge mono-alkyl anionic surfactants usually form small globular micelles. In general, the first phase for surfactants with short alkyl chains (at the L_1 boundary) is a direct hexagonal phase (H_1) followed by bicontinuous cubic (V_1) and lamellar phases (L_α). H_1 and L_α phases are easily determined because of their birefringence, in contrary to the bicontinuous phase. Moreover, H_1 phase has higher viscosity than L_α phase. Concerning hydrotropes, the molecules self-aggregate to produce the operating assembly beyond the minimal hydrotropic concentration. The molecular organization of hydrotropes has been investigated several times[217,220].

In the case of PMD-Succinate, the binary phase diagram in Graph. 7.4 shows a pattern different from that of typical anionic surfactants. In fact, the diagram is divided into two areas (see Fig. 7.1).



Graph. 7.4: Binary phase diagram of PMD-Succinate.

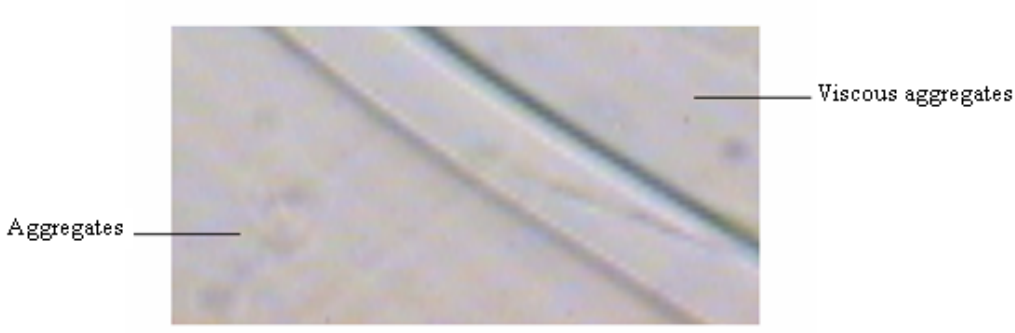


Fig. 7.1: Microscope photography.

The first area consists of aggregates and the hypothesis of small micelles formation is considered. Nevertheless, Dynamic Light Scattering (DLS) measurements have been carried out on different PMD-Succinate concentrations and no spherical structure was observed. The hypothesis of small micelles formation is then also rejected. Srinivas *et al*[217] have reported the crystal structures of several hydrotropes in solid phase by X-ray diffraction. These solid compounds show open layer assemblies, reminiscent of lamellar liquid crystals of a surfactant, consisting of alternating hydrophobic clusterings of the non-polar regions adjacent to the ionic or polar regions that are knitted together in a two-dimensional network. Moreover, Pal *et al.*[218] have investigated the microstructures of sodium n-butyl benzene sulfonate that aggregates in aqueous solutions and displays ellipsoidal forms. According to the previous publications and the PMD-S structure, which is very close to the geometry of classical hydrotropes, PMD-Succinate may form ellipsoidal open-layer assemblies.

The second area consists of one highly viscous and viscoelastic phase. The viscous phase could be a cubic phase because of its non-birefringence and its high viscosity. Non-spherical air bubbles can be observed under microscope as shown in Fig. 7.2. The presence of such bubbles is typical of one cubic phase. The formation of this phase may be explained by the association of the polar head groups. At high concentrations, the sodium ions may stay in the vicinity of the hydrotrope aggregates by neutralizing the charge more effectively.

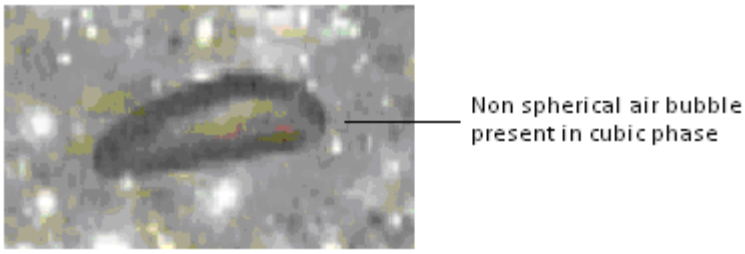
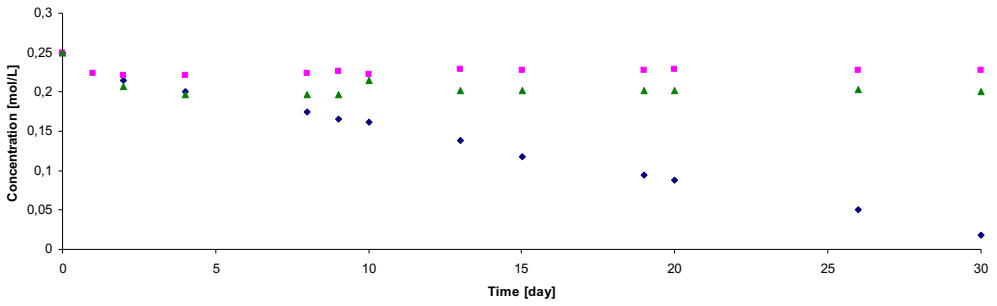


Fig. 7.2: Non-spherical air bubbles in the cubic phase.

As shown in Graph. 7.4, the measurement of viscosity confirms the two areas. In the first area, the viscosity is low and the solutions are Newtonian fluid. In the second area, the viscosity is high and the solutions are dilatant fluids. Furthermore, with the increase in concentration, the aggregates become more compact by increasing the viscosity.

Hydrolysis

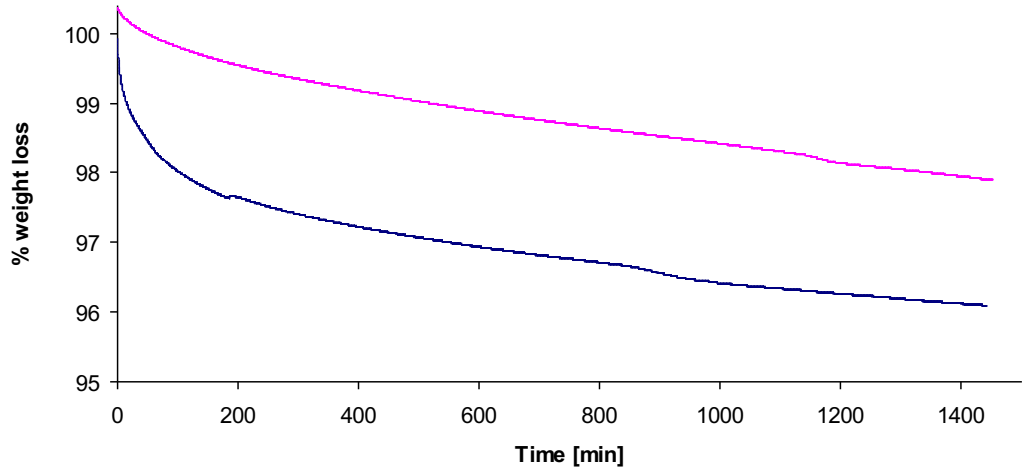
Ester functions are sensitive to hydrolysis in acidic or basic mediums. The stability of PMD-Succinate in aqueous solution was studied at 20°C at various pH values (5, 7 and 11) for 30 days. Graph. 7.5 shows the stability of PMD-S at pH 5; 91% of PMD-S remains unchanged. This high stability can be explained by the low solubility of the ester, which is both under a salt and an acid form. At pH 7, 80% of PMD-Succinate remains unchanged due to the presence of aggregates protecting the ester function. The half-life of PMD-S at pH 11 is around 16 days. In conclusion, PMD-Succinate shows high stability in basic and in acid mediums and may consequently be stored at suitable pH values, both in a pure form or in aqueous solutions.



Graph. 7.5: Hydrolysis evolution of PMD-Succinate at pH 5 (■), 7 (▲), and 11 (◆).

Thermo-gravimetric analysis

TGA analysis were settled to measure the amount and rate of change in the mass of pure PMD and PMD-Succinate as a function of the temperature of the human skin surface (33°C). As shown in Graph. 7.6, the weight loss of PMD-S is around 4% after 24 hours in comparison to 2% for pure PMD. These results confirmed the same behaviour between the two repellent actives in term of evaporation stability, which is relatively negligible and thus goes along with a long-lasting repellent property for PMD-S.



Graph. 7.6: Thermo-gravimetric analysis of PMD-S (——) and PMD (——) at 33°C.

PMD-S solubility

PMD-Succinate has a high solubility in water, which is over 250g/litre at 25°C. In conjunction with its protective activity against mosquitoes, this solubility makes the active useful for aqueous formulations, since the compounds used in repellents are in principle water-insoluble or water-soluble only to a limited extent.

Table 7.3 shows the solubility in water of PMD-S in comparison to conventional active ingredients used in insect repellents.

Active ingredient	Solubility in water (g/litre)
DEET	1 g/litre at 25°C
KBR 3023	8.2 g/litre at 25°C
PMD	0.29 g/litre at 25°C
IR 3535	70 g/litre at 25°C
PMD-S	> 250 g/litre at 25°C

Table 7.3: Water solubility of different repellent actives.

PMD-S has also a high solubility in chloroform, acetone, diethylether, ethanol, methanol, dimethylsulphoxide and acetonitrile, but only a restricted solubility in alkanes, such as for example pentane and hexane.

Ternary diagram

Following the previous results, PMD-Succinate can be considered as a hydrotrope and thus could increase the solubility in water of hydrophobic compounds such as pure PMD. As shown in Fig. 7.7, a ternary diagram has been established for this purpose using the three-component system PMD/PMD-S/Water. **L** marks the region of clear single-phase mixtures and **N** the region of multi-phases mixtures.

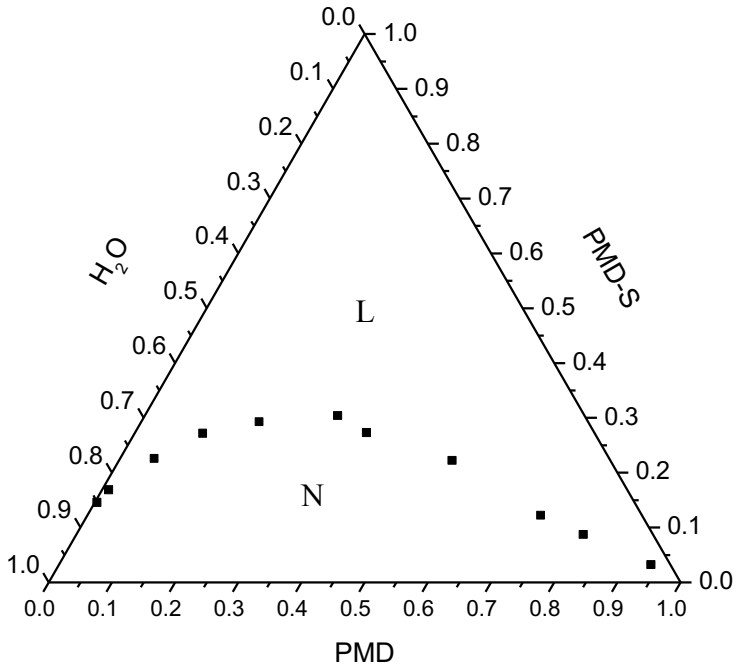


Fig. 7.7: Schematic ternary phase diagram of the PMD/PMD-S system at 25°C. Compositions are in weight ratio. L is the domain of existence of the clear and monophasic solutions. N is the domain of existence of the biphasic solutions.

As can be seen from the phase diagram, the two-component system H_2O/PMD (shown by the lower axis), apart from extreme values in the region of almost 100% H_2O or almost 100% PMD, still consists of several phases. However, the formation of single-phase mixtures can be achieved by the addition of an adequate quantity of PMD-S. A simple example of an aqueous composition of mosquito repellent was obtained by mixing at room temperature the components listed in the following Table 7.4, with the indicated ratio.

Component	**Wt.%**
Water	68.85
PMD-S	25
PMD	5
Fragrances	0.5
Sodium benzoate	0.4
Potassium sorbate	0.15
Tocopherol	0.10

Table 7.4: Example of a mosquito repellent formulation composed of PMD-S/PMD/Water.

7.5. Conclusion

In this study, the general properties of PMD-Succinate were reported and confirmed its high surface activity with a MHC similar to other hydrotropes (0.29M). The PMD-S is relatively stable to hydrolysis and a binary phase diagram showed the different structure assemblies. Due to its high solubility in water, PMD-S allows the formulation of concentrated insect repellent in aqueous mediums without the addition of surfactants or alcoholic co-solvents. Furthermore, PMD-Succinate may be used to increase the solubility of current insect repellent actives, such as DEET, KBR 3023, IR3535, as a result of which, the use of organic solvents and/or surfactants in the formulation are also avoided. An example of a PMD-S/PMD (25/5 wt.%) formulation is given in this study. By combining one or more active ingredients in the same formulation it would be possible to reach a higher protection time and/or to extend the protection on different species of mosquitoes by using the same product.

Chapter 8

A COMPARATIVE STUDY OF CYTOTOXIC EFFECTS OF 5 MOSQUITO REPELLENT ACTIVES, IR3535, PMD, DEET, KBR3023 AND NOVEL PMD-SUCCINATE (PMD-S), ON KERATINOCYTE AND HELA CELLS

8.1. Abstract

The MTT assay has been used to determine the cytotoxicity of mainly used mosquito repellent actives, IR3535, PMD, DEET, KBR 3023 in comparison to the new molecule PMD-Succinate (PMD-S). The study focused on Keratinocyte and HeLa cells in order to investigate the potential skin and eye irritation. The 50% inhibition concentration (IC_{50}) for MTT reduction was calculated. Data analysis using the ANOVA routine demonstrated that PMD-S is significantly less toxic than DEET or KBR 3023 for keratinocytes and comparable to PMD and IR3535. For Hela cells, PMD-S reaches results similar to IR3535, DEET and KBR 3023. Finally, PMD-Succinate has a skin and eye tolerance similar to the commonly used IR3535 and thus supports its applicability in customer end-repellents.

8.2. Introduction

Mosquitoes kill significantly more people (700 000) every year than any other animals including other humans by spreading diseases like dengue, yellow fever, Chikungunya, West Nile virus[221]. By far, the deathliest diseases caused by mosquitoes is malaria, which killed an estimated 440 000 people in the year 2012[222]. Prevention and control rely on reducing the number of infected people by using a personal protection like skin repellent products[221,223]. Chemical (synthetic) and natural (plant-derived) insect repellents are currently available on the market with an effective reduce of the risk of infection and discomfort created. The active *N,N*-diethyl-*m*-methylbenzamide (DEET) is for instance used by 30% of the US population[224], whereas it has been implicated in severe neurotoxicity factors in Human[99,225,226]. 1-piperidincarboxylid acid 2-(2-hydroxyethyl)-1-methylpropylester (KBR 3023) is also used in the well-known Autan® from S.C. Johnson. *Para*-Menthane-3,8-diol (PMD) is often cited as a natural repellent active but is mainly produced from synthetic (+)-citronellal over a well-defined process[75]. IR3535 (ethyl 3-[(acetyl)(butyl)amino]propanoate) is a chemical repellent available in Europe for 30 years and sold through a wide range of products[227]. A new repellent active developed in Chapter 6 so called PMD-Succinate (PMD-S) has several advantages in comparison to other actives mentioned previously: a high repellent activity, a solubility in water and it is synthesized from a natural source. Apart from the efficacy of these repellents, consumers are more and more concerned in relation to their own health when applying synthetic actives on their skin. Suspicions survive somewhat due to opaqueness comprehension from the customer on the reliable toxicity aspect of these consumable products. For this reason, it is important to establish an easy way to estimate the potential toxicity of such repellent actives.

Animal testing of cosmetic ingredients in the EU has been prohibited since 2009 and cytotoxicity tests on established cell lines represent a good alternative[228] for toxicity tests. The cytotoxicity of the 4 commonly used repellents (IR3535, PMD, DEET, KBR 3023) was measured and compared to the toxicity of the new active PMD-S in order to determine its potential integration into marketed repellent products. Keratinocyte cells (SK-MEL-28) were used to report the skin compatibility. Keratinocyte cells are the major constituent of the superficial skin layers (epidermis) and have been reported to provide a

useful prediction of skin damages. HeLa cells namely cervix carcinoma are well-known cell lines that are used to assess the cytotoxicity of chemical compounds[229-232] and reportedly show a great reproducibility and a good correlation with *in vivo* results[233]. The cytotoxicity test using MTT on HeLa cells was evaluated as an alternative method to the Draize eye irritation test (Draize test) to predict the eye irritation aspect[234-237].

The yellow tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) is widely used to determine cell viability in cell proliferation and cytotoxic assays. MTT is reduced by metabolically active cells to form an insoluble purple formazan product. The IC_{50} value (concentration the test substance that lowers MTT reduction by 50% compared with the untreated control) was calculated from the absorbance.

8.3. Experimental procedures

8.3.1. Material

Repellent actives

para-Menthane-3,8-diol (PMD; Takasago, France; ratio cis/trans 62/38), *N,N*-diethyl-*m*-toluamide (DEET; Sigma-Aldrich, Germany; grade: 95%), 1-piperidincarboxylid acid 2-(2-hydroxyethyl)-1-methylpropylester (KBR3023; Saltigo, Germany: grade: 99%), 3-[N-butyl-N- acetyl]-aminopropionic acid (IR3535; Merck, Germany) were used as received. PMD-Succinate (PMD-S) was synthesized according to the process previously developed in Chapter 6. Also, ethanol (EtOH; J.T. Baker, Holland; assay min. 99.9% (v/v)) was used. The PMD, DEET, KBR3023, IR3535 solutions were prepared by dissolving weighed amount of repellents in ethanol whereas PMD-S was solubilized in Millipore water.

Hela Cell Lines Hela Cells were distributed by CLS - Cell Lines Service (Eppelheim, Germany). The HeLa cells were cultured in Earles's Minimum Medium (MEM) provided by Biochrom AG (Berlin, Germany) containing 0.85 g/L $NaHCO_3$ supplemented with

FCS (10%), l-glutamine (2 mM), NEA (1%), Amphotercin B (0.4 µg/mL), and Penicillin G/Streptomycin sulfate (100 u/mL).

Keratinocytes Cell Lines Keratonocytes cells were obtained from CLS - Cell Lines Service (Eppelheim, Germany). The Keratinocytes cells were grown in modified Earle's medium (MEM) containing 2 mM L.glutamine, 0.1 mM non-essential amino acids (NEA), 1 mM Na pyruvate and 10% fetal bovine serum (FBS). The Keratinocyte cells were cultured in a specific medium using a Dublbecco's Modified Eagle Medium (4.5 mg⁄L D-glucose, L-glutamine, and 25 mM HEPES buffer, but no sodium pyruvate or phenol red) provided by Invitrogen GmbH (Karlsruhe, Germany) and Ham's F-12 liquid medium (1.176 g⁄L NaHCO3, stable L-glutamine and low endotoxin) purchased from Biochrom AG (Berlin, Germany) in a ration 1:1 supplemented with FCS (10%).

8.3.2. Cell viability assay

The IC_{50} of the repellent actives for Keratinocyte and HeLa cells was determined using 3-(4,5-Dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay as previously described by Mosmann[238]. Cells seeded (2.5 x 10^3 cells/well) in 96-well flat-bottom culture plates were incubated for 72h at 37°C with different concentrations of actives in the culture medium. Ethanol plates were also performed as a control. After the initial treatment, a standard MTT-dye assay (15 μl of MTT - 5 mg/mL) was added to measure viable cells. The medium was removed gently after a subsequent 4 hours incubation. 150 μl of 10% SDS solution was added on each well. The plates were kept in a dark room overnight and the optical density was measured at 560 nm using a microplate reader. The cytotoxicity is calculated from the concentration-response curve as the concentration of the active lowering MTT reduction by 50% (IC_{50} in μg/ml) compared to the control. 5 replicate plates were used to determine the average IC_{50} for each active.

8.3.3. Statistical analysis

Data reported as the mean IC_{50} were subjected to an analysis of variance (ANOVA) and Post-Hoc T-test ($P<0.05$) using the software IBM SPSS Statistics (version 22 for

windows). This statistical analysis was used to check the reliability of the results obtained from the assay.

8.4. Results and discussion

8.4.1. Keratinocyte cells

The five repellent actives were tested for their cytotoxic potential against SK-MEL-28 cells. The cell viability was determined by the tetrazolium MTT dye reduction in the cell culture system[238]. The IC_{50} values are reported in Table 8.1 and Figure 8.1.

Repellent active	Keratinocyte cells [IC_{50} μg/mL]	HeLa cells [IC_{50} μg/mL]
IR3535	17.51 ± 2.64	12.49 ± 1.30
PMD	14.18 ± 5.24	19.82 ± 3.37
DEET	8.10 ± 3.41	12.39 ± 1.89
KBR3023	9.82 ± 2.39	9.71 ± 2.25
PMD-S	**18.56 ± 1.56**	**9.28 ± 0.78**

Table 8.1: The IC_{50} values with Keratinocyte and HeLa cells (concentration of active repellent that lowers MTT reduction by 50%) of PMD-S compared to IR3535, PMD, DEET, KBR3023.

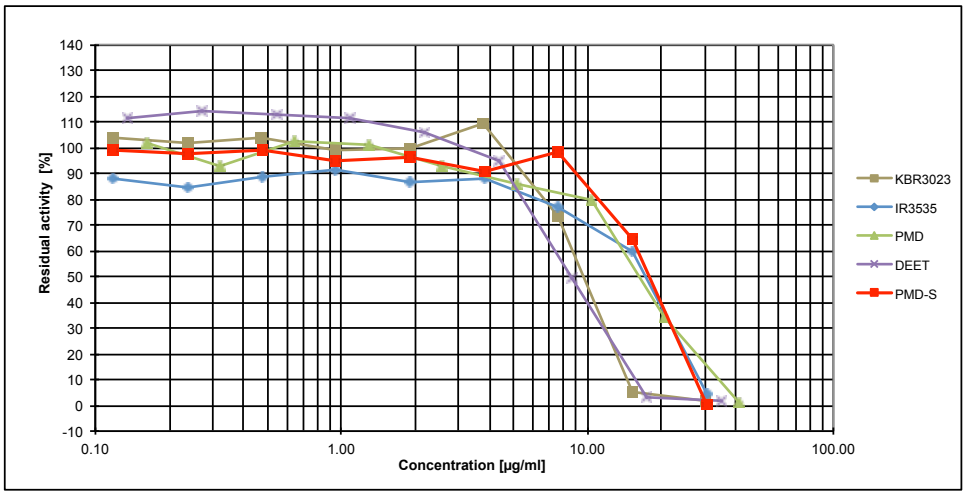


Fig. 8.1: IC_{50} values of KBR 3023, IR3535, PMD, DEET, PMD-S obtained after 72 h of incubation with SK-MEL-28 cells.

Against SK-MEL-28 cells, the new repellent active PMD-Succinate displayed the lowest cytotoxic activity with an IC_{50} value of 18.56 µg/mL. PMD-S and IR3535 (IC_{50} = 17.54 µg/mL) showed similar activity as well with PMD (IC_{50} =14.18 µg/mL) with no significant difference between the three compounds. KBR 3023 and DEET displayed the highest cytotoxicity effect with IC_{50} values of 9.82 and 8.10 µg/mL respectively. ANOVA statistics calculations (See Table 8.2) showed a significant difference between KBR 3023 / DEET and the new repellent PMD-S.

In studies using laboratory animals, LD_{50} (Lethal Dose) is one way to measure the short-term poisoning potential (acute toxicity) of a chemical. Following the safety data sheets of the different repellent active, Table 8.3 gives the category for acute dermal and ocular toxicity of the 4 commonly used repellent compounds.

	IR3535	**PMD**	**KBR 3023**	**DEET**
DERMAL	III	IV	III	IV
OCULAR	II	I	III	II

Table 8.3: Toxicity classification of acute dermal LD_{50} and primary eye irritation for 4 repellent actives:

- Category I is Highly toxic and Severely irritating,
- Category II is Moderately toxic and Moderately irritating,
- Category III is Slightly toxic and Slightly irritating,
- Category IV is Practically non-toxic and not an irritant.

According to the acute dermal LD_{50} given by the manufacturers in Table 8.3, we can establish the following ranking from the practically non-toxic active (Category IV) to the highly toxic one (Category I): PMD > DEET >IR3535 > KBR 3023. Following the IC_{50} values on SK-MEL-28 cells in Table 8.1, the same ranking can be carried out by introducing the new repellent active: PMD-S > IR3535 > PMD > KBR 3023 > DEET. No sufficient correlation could be established to explain the differences in the classification between the two toxicity determination methods.

Additionally, the studies on the solubility and the structure of the different molecules that could influence the toxicity did not reveal a sufficient conclusion to explain the different rankings. In conclusion, it can be seen that the skin tolerance of the new active PMD-S is

particularly good with the higher IC_{50} value in the same range of the commonly used IR3535.

Repellent active	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
IR3535	17.508	2.638	1.180	14.233	20.783	15.670	22.060
PMD	14.186	5.239	2.343	7.681	20.691	10.640	23.000
DEET	8.100	3.414	1.527	3.861	12.339	4.460	12.570
KBR3023	9.820	2.395	1.071	6.846	12.794	6.580	12.970
PMD-S	**18.562**	**1.565**	**0.700**	**16.619**	**20.505**	**15.800**	**19.660**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	423.848	4	105.962	9.767	**0.00015**
Within Groups	216.971	20	10.849		
Total	640.818	24			

Repellent active		Mean Difference (I-J)	Std. Error	Sig.
IR3535	PMD	3.322	2.083	0.517
	DEET	9.408	2.083	0.002
	KBR3023	7.688	2.083	0.011
	PMD-S	-1.054	2.083	0.986
PMD	IR3535	-3.322	2.083	0.517
	DEET	6.086	2.083	0.058
	KNR3023	4.366	2.083	0.260
	PMD-S	-4.376	2.083	0.258
DEET	IR3535	-9.408	2.083	0.002
	PMD	-6.086	2.083	0.058
	KBR3023	-1.720	2.083	0.920
	PMD-S	-10.462	2.083	0.001
KBR3023	IR3535	-7.688	2.083	0.011
	PMD	-4.366	2.083	0.260
	DEET	1.720	2.083	0.920
	PMD-S	-8.742	2.083	0.004
PMD-S	IR3535	1.054	2.083	0.986
	PMD	4.376	2.083	0.258
	DEET	**10.462**	**2.083**	**0.001**
	KBR3023	**8.742**	**2.083**	**0.004**

Table 8.2: One-way ANOVA and multiple comparisons T-test ($P<0.05$) of repellent actives with Keratinocytes cells.

8.4.2. HeLa cells

The five repellent actives were tested for their cytotoxic potential against HeLa cells. The cell viability was determined by the tetrazolium MTT dye reduction in the cell culture system[238]. The IC_{50} values are reported in Table 8.1 and Figure 8.2.

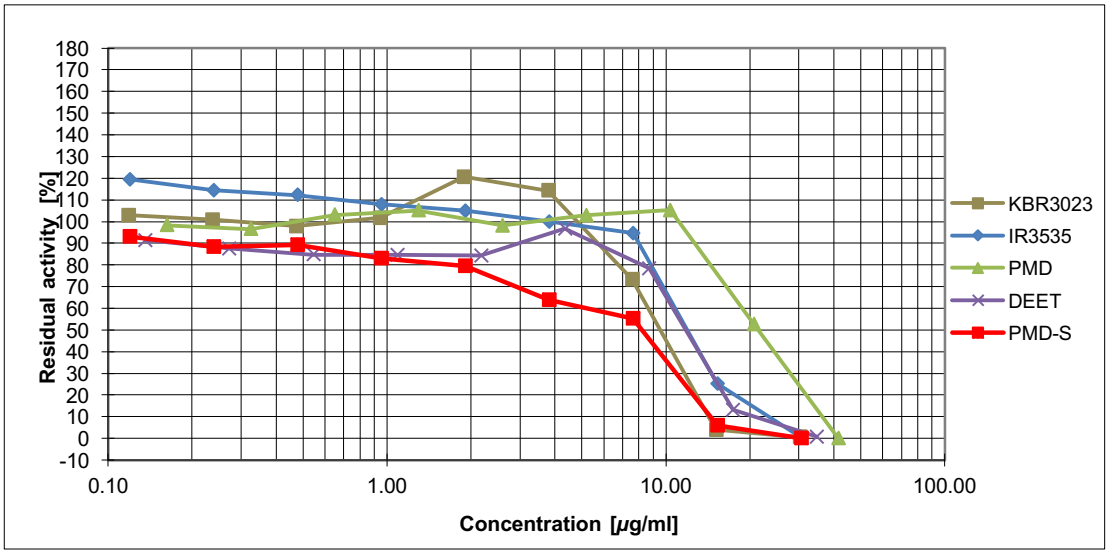


Fig. 8.2: IC_{50} values of KBR 3023, IR3535, PMD, DEET, PMD-S obtained after 72 h of incubation with HeLa cells.

The results of cytotoxicity on HeLa cells clearly showed the superior cytotoxic properties of PMD-S with an IC_{50} value of 9.28 µg/mL. Data analysis using the ANOVA routine (See Table 8.4) demonstrates that the values obtained for KBR 3023 (IC_{50} = 9.71 µg/mL), DEET (IC_{50} = 12.39 µg/mL) and IR3535 (IC_{50} = 12.49 µg/mL) did not show any significant difference with PMD-S. However, PMD displayed the lowest cytotoxic activity with an IC_{50} value of 19.82 µg/mL, which is notably different to PMD-S.

The same ranking with data on primary eye irritation was prepared in Table 8.3 using the safety data sheets from the manufacturers: KBR 3023 > IR 3535 > DEET > PMD. The IC_{50} tests on HeLa cells in Table 8.1, revealed following cytotoxic ranking for the repellent actives: PMD > IR3535 > DEET > KBR 3023 > PMD-S. Again, no relationship can explain the difference in classification between these two methods of testing for the eye irritation consideration.

PMD-S acts as a hydrotrope and may be compared to others anionic surfactants despite its general structure. It has been established that ionic surfactants show higher cytotoxicity than the non-ionic ones[239]. PMD-S is the only salt structure in comparison to the others repellent actives and then displayed the higher cytotoxicity activity on Hela cells but still lies in the same order of magnitude than IR3535, DEET or KBR 3023.

Repellent active	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
IR3535	12.498	1.309	0.585	10.872	14.124	10.510	14.120
PMD	19.825	3.374	1.509	15.636	24.014	15.070	24.580
DEET	12.392	1.897	0.848	10.036	14.748	10.860	15.420
KBR3023	9.718	2.256	1.009	6.916	12.520	6.600	12.460
PMD-S	**9.280**	**0.782**	**0.350**	**8.309**	**10.251**	**7.900**	**9.830**

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	357.406	4	89.352	19.946	**.000**
Within Groups	89.594	20	4.480		
Total	447.000	24			

Repellent active		Mean Difference (I-J)	Std. Error	Sig.
IR3535	PMD	-7.327	1.339	.000
	DEET	0.106	1.339	1.000
	KBR3023	2.780	1.339	.268
	PMD-S	3.218	1.339	.155
PMD	IR3535	7.327	1.339	.000
	DEET	7.433	1.339	.000
	KBR3023	10.107	1.339	.000
	PMD-S	10.545	1.339	.000
DEET	IR3535	-0.106	1.339	1.000
	PMD	-7.433	1.339	.000
	KBR3023	2.674	1.339	.303
	PMD-S	3.112	1.339	.178
KBR3023	IR3535	-2.780	1.339	.268
	PMD	-10.107	1.339	.000
	DEET	-2.674	1.339	.303
	PMD-S	0.438	1.339	.997
PMD-S	IR3535	-3.218	1.339	.155
	PMD	**-10.545**	**1.339**	**.000**
	DEET	-3.112	1.339	.178
	KBR3023	-.43800	1.33861	.997

Table 8.4: One-way ANOVA and multiple comparisons T-test (P<0.05) of repellent actives with Hela cells.

8.5. Conclusion

The cytotoxicity of 4 well known repellent actives (IR3535, PMD, DEET, KBR3023) and a new molecule PMD-S was evaluated using MTT on Keratinocyte and HeLa cells in order to examine their potential use in repellent formulations against mosquitoes. PMD-S displayed the lowest cytotoxicity effects for SK-MEL-28 cells (skin compatibility) but the highest properties for Hela cells (eye irritation). There was no good

correlation between the cytotoxicity IC_{50} in vitro and the reported data on dermal acute LD_{50} and primary eye irritation provided by the manufacturers with the marketed repellent actives. However, as a primary conclusion for toxic considerations, the skin tolerance of PMD-S is particularly good and the expected eye irritation is indeed higher than the current insect repellents.

Chapter 9

IMPROVED SYNTHESIS OF THE MOSQUITO REPELLENT ACTIVE PMD-SUCCINATE (PMD-S) WITH AN ECO-FRIENDLY METHOD

9.1. Abstract

A simple and efficient synthesis of the new repellent mosquito active Sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate (PMD-Succinate) has been developed. *Para*-Menthane-3,8-diol (PMD) was reacted with succinic anhydride under a conventional mixing process at 100°C for 48h. The purification method involved an ion-exchange resin (Amberlite® IRA-900) able to separate PMD-Succinate to impurities with a yield of 58.24 % and to collect non-reacted PMD for recycling. The reactions were characterized by ^{1}H-NMR and ESI-MS. The advantages of this new method include simple operational process, no toxic solvent used, safety and environmental benignancy.

9.2. Introduction

In a previous study (See Chapter 6), *para*-Menthane-succinate (PMD-S) has been found to possess a high repellent action against *Aedes aegypti* mosquitoes, statistically comparable to the market reference *N,N*-diethyl-*m*-methylbenzamide (DEET). The

method of synthesis suffered from some drawbacks such as harsh reaction conditions, low yield and the use of toxic solvents.

The recent interest in green chemistry has posed a new challenge for organic synthesis in that new reaction conditions need to be found, which reduce the use of hazardous organic solvents, shorter the synthesis and ensure atom economy or efficiency[240-242]. The prevention of waste can be achieved if most of the reagents and the solvent are recyclable. For example, catalysts and reagents such as acids and bases that are bound to a solid phase can be filtered off, and can be regenerated (if needed) and reused in a subsequent run[243,244]. In the production of chemical products on very large scale, heterogeneous catalysts and reagents can be kept stationary while substrates are continuously added and pass through to yield a product that is continuously removed (for example by distillation). A key point is the choice of solvent, as this is often the main component of a reaction system by volume. The solvent of choice for green chemistry is water, which is a non-toxic liquid but with limited chemical compatibility[245-247]. Chemical reactions running under neat conditions (no solvent) and in a supercritical CO_2 medium can also be considered as green choices[248]. Other possible improvements can be suggested, such as for example replacement of benzene by toluene (as a less toxic alternative)[249], or the use of solvents that can be rapidly degraded by microorganisms[250]. Several ways for achieving green processes include new and innovative methodologies using alternative energy inputs, such as microwave[251-253], mechanochemical mixing[254-256], high-speed ball milling[257] or sonication[258,259]. In total, there is a great demand for developing facile, efficient and non-polluting synthetic procedures to build cosmetic or pharmaceutical actives.

In this study, we were interested in an efficient and environmental-friendly protocol for the synthesis of the new repellent active PMD-Succinate by using non-toxic solvents and by reducing the waste of reagents.

9.3. Experimental procedures

9.3.1. Chemical

Oxolane-2,5-dione (Succinic anhydride), Trichloromethane (Chloroform), Amberlite® IRA-900 chloride form, Ethoxyethane (Diethyl ether), were obtained from Sigma Aldrich (Germany). 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Takasago, France) was used as received. Hydrochloric acid, Ethyl alcohol (Ethanol), Sodium hydroxide and Phosphomolybdic acid were purchased from Merck (Germany).

9.3.2. Analytical equipment

NMR spectroscopy ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 300 instrument using D_2O or $CDCL_3$ as solvent.

Mass spectrometry Mass spectra were carried out with a Waters Xevo-QT using electrospray ionization.

Detection on Thin Layer Chromatography (TLC) Plates Standard solutions were prepared and spotted on the TLC plates. Plates were air-dried and subjected to TLC using ether as mobile phase. After development, the plates were dried and sprayed with Phosphomolybdic acid in ethanol. The plates were then heated for several minutes with a heat gun and the spots were recorded.

9.3.3. Synthesis of PMD-S

In a single neck round bottom flask, PMD (1g, 0.0058 mole) was added to a preferred molar ratio (see Table 7.1, Entry n°5) of succinic anhydride (0.29g, 0.0029 mole). The flask is placed in an oil bath and covered up to the neck. The reaction medium is stirred for 48 hours at 100°C. PMD melts at 60°C and solubilizes succinic anhydride at 90°C. The solubilisation process takes between 4 to 5 hours. The reaction medium is then solubilized in chloroform (20 mL). The solution is washed two times (30 mL) with an

acidic solution (10^{-1}M HCl). The organic phase is removed under vacuum and the crude oil is collected (m=1.17g). The oil is composed of PMD-S (0.75g, 0.278 mol), PMD (0.39g, 0.229 mol), PMD-bis-S (0.015g, 0.004 mol).

9.3.4. Separation of PMD-S and PMD

Preparation of the Amberlite® IRA-900 The resin was cleaned before use. In a 35 cm height column, the Amberlite resin (60 to 120g as a function of the diameter) is conditioned with an acidic solution (10^{-1}M HCl). 200 ml of an acidic solution (10^{-1}M HCl) are then added drop-wise to the column. A volume of distilled water is passed through the column. As long as the pH at the outlet of the column is not close to neutrality, distilled water is added. Finally, the resin is washed with ethanol (250 mL).

Preparation of PMD-S The acidic form of PMD-S must be neutralized for separation. The previous crude oil is solubilized in distilled water and a sodium hydroxide solution (10^{-4} M) is progressively added to reach pH 7. A very hygroscopic white solid is obtained after the evaporation of water. The reaction mixture is then solubilized in 30 mL of ethanol.

Separation method

First step: The previous solution is added drop-wise to the column. Additionally, 100 ml of pure EtOH are passed through the column. The collected fractions at the end of the column are evaporated. TLC plates (ether as eluent) are used to check the presence of PMD and PMD-S. Two more volumes of EtOH are passed through the column. Salt crystals are obtained in the solution. After evaporation, the crude is solubilized in $CHCl_3$, filtered and again evaporated. TLC plates (ether as eluent) and ^{1}H NMR investigations indicated that the oil is composed of pure PMD (0.541g, 0.003 mole). The resin is washed with a 100 mL solution of ethanol/distilled water (70/30) in order to remove undesirable traces of PMD.

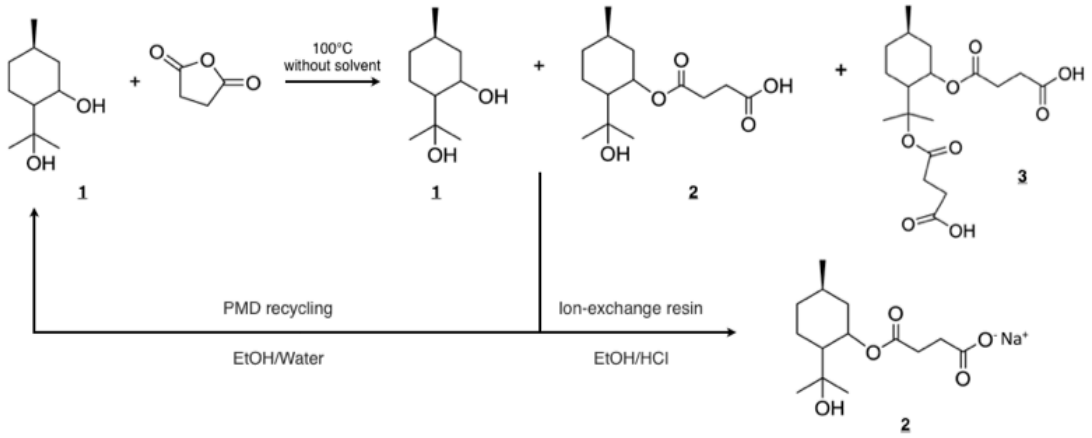
Second step: The eluting solution is a mixture of ethanol/acidic solution (HCl 10^{-1}M) (ratio 9:1). Several volumes (100 mL) of the solution are passed through the column until there is no more PMD-S coming out. TLC plates (ether as eluent) and ^{1}H NMR

investigations indicated that the oil is composed of PMD-S (0.46g, 0.0017 mol) and traces of PMD-bis-S (0.04g, 0.0001 mol). Yield: 58.24%

9.4. Results and discussion

9.4.1. Synthesis of PMD-S

In Chapter 6, we reported the synthesis of the new repellent active Sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate so called PMD-Succinate (**2**). It was obtained by esterification of *para*-Menthane-3,8-diol (**1**) with succinic anhydride using large volumes of solvents and an unsatisfactory yield was reached at the end. In this study, these problems were avoided with a modification of the reaction route by using an easy preparation of (**2)** without conventional solvents. Thus, considering that succinic anhydride is soluble in PMD at 100°C, PMD-S was obtained by a simple mixing process for several hours, as shown in Scheme 9.1



Scheme 9.1: Esterification of *para*-Menthane-3,8-diol (**1**) with succinic anhydride. Synthesis of *para*-Menthane-Succinate (**2**) and *para*-Menthane-bis-succinate (**3**) (side product).

The influence of the reaction conditions on the synthesis of *para*-Menthane-3,8-diol (**1**) with succinic anhydride is summarized in Table 9.1. The composition of the mixture at the end of the reaction was determined by mass spectrometry (negative mode ESI) from the characteristic ion area ratios. The values of the ionization coefficients of the different

compounds present in the medium, PMD (**1**), PMD-S (**2**), PMD-bis-Succinate (**3**), were previously determined.

	Molar ratio between the reagents			Relative concentration of the products[a]		
Entry	**PMD (1)**	**Succinic anhydride**	**Time (h)**	**PMD-S (2)**	**PMD (1)**	**PMD-bis-S (3)**
1	1	3	24	100	56	50
2	1	1.5	24	100	70	28
3	1	1	24	100	78	21
4	1	0.5	24	100	68	5
5	1	0.5	48	100	50	2
5 bis[b]				100		8.7

Table 9.1: Influence of reaction conditions on the synthesis of PMD-succinate (**2**). Reaction temperature: 100°C. [a] Relative concentration of **1** and **3** compared to **2**, with 100 as a base for **2** determined by mass spectrometry. [b] Relative concentration of **3** compared to **2**, with 100 as a base for **2** after the second elution on the ion-exchange resin.

PMD (**1**) and PMD-S (**2**) can easily be separated by extraction or by chromatography on an ion-exchange resin as explained in section 9.4.2. However, the separation of PMD-S from PMD-bis-S (**3**) is more difficult. Different operating conditions were set up to minimize the formation of (**3)** during the reaction.

As shown in Table 9.1, the conversion of PMD (**1**) is not total. In stoichiometry condition or excess of succinic anhydride, the proportion of PMD-bis-S (**3**) compared to PMD-S (**2**) is high (from 10.5 to 24.3%, Table 9.1, Entry **1**, **2**, **3**). By decreasing the molar ratio to 1:0.5 and with a longer reaction time (48h) the proportion of PMD-bis-S (**3**) drastically decreased to reach 1.3%. From previous results, the optimum reaction conditions were: *para*-Menthane-3,8-diol (**1**) (5.8 mmol), Succinic anhydride (2.9 mmol) at 100°C for 48h (Table 9.1, Entry **5**). Under this condition, several experiments were made to separate PMD-S (**2**) from PMD (**1**). and PMD-bis-S (**3**).

9.4.2. Separation of PMD-S and PMD with an ion-exchange resin

PMD-S (**2**) in its carboxylate form can be attached to an anion-exchange resin while PMD (**1**) is not retained. The fixed PMD-S (**2**) is then withdrawn in an acid medium or in the presence of salts. In this study, a chloride ion exchange resin (Amberlite® IRA-900) was used[260,261].

In order to confirm the separation technique, preliminary tests were performed with a mixture solution of pure PMD/PMD-S. In a first step and under optimized conditions (see part 9.3.4), PMD (**1**) was separated quantitatively by a first elution using EtOH, while PMD-S (**2**) remained on the resin. On a second step, PMD-S (**2**) and PMD-bis-S (**3**) were withdrawn from the resin by elution of EtOH/HCl 10^{-1} M (90/10). The separation was followed by TLC and mass spectroscopy (see Table 9.1, Entry **5bis**).

The following sequence of elutions gave a satisfactory separation of pure PMD (**1**). From the initial concentration, 54.1% of pure PMD was recovered and could be entered into a new recycling scheme thus limiting the production of waste. The yield of the complete synthesis of pure PMD-S (**2**) reached 58.24%.

9.5. Conclusion

In conclusion, we have described a practical, catalyst-free and solvent-free method to synthesize Sodium 4-((2-(2-hydroxypropan-2-yl)-5-methylcyclohexyl)oxy)-4-oxobutanoate, the so-called PMD-Succinate. The merits of this protocol such as easy and eco-friendly operations, recycling materials and a high yield (58.24%), make it an attractive approach to produce the new mosquito repellent active at larger scales.

PART III

STUDIES OF 19 CORSICAN ESSENTIAL OILS

Chapter 10

REPELLENT STUDIES WITH *AEDES AEGYPTI* MOSQUITOES AND HUMAN OLFACTORY TESTS ON 19 ESSENTIAL OILS FROM CORSICA (FRANCE)

10.1. Abstract

In order to reduce the risk of getting infected with any epidemic disease transmitted by mosquitoes, repellent products are often used to protect populations at risk. The repellent potential of 19 essential oils from Corsica Island (France) was evaluated in a bio-assay with *Aedes aegypti*, in order to assess the "space repellent" properties of these oils. *Lavendula stoechas*, *Helichrysum italicum* (leaves) and *Laurus nobilis* oils showed a capability of reducing the attractivity of a human finger for yellow-fever mosquitoes in a Y-tube olfactometer. In addition to the behavioral studies on mosquitoes, two tests on the olfactive perception of these 19 oils were performed, involving 25 female and 25 male Human volunteers. The studied aspects were the "hedonic dimension" of these oils on the one hand, and their acceptance as a final fragrance for a repellent formulation on the other. The experiments yielded promising results for three oils, *Calamintha nepeta*, *Laurus nobilis*, *Rosmarinus officinalis* concerning both aspects, with minor differences between male and female participants. *Laurus nobilis* oil was the only oil tested fulfilling both properties: a spatial repellent effect on *Aedes aegypti* and the acceptance by the volunteers for its integration in a repellent product. Thermogravimetric analysis showed that *Calamintha nepeta* oil has a slower evaporation rate in comparison to the *Laurus nobilis* and *Rosmarinus officinalis*.

10.2. Introduction

Aedes aegypti and *Aedes albopictus* are two important vectors of arthropod-borne-viruses, such as yellow-fever, dengue- and chikungunya-fever, and cause world-wide outbreaks of severe epidemic diseases every year[78]. 350-500 million cases of malaria, which is transmitted by *Anopheline* mosquitoes, are reported annually and over one million people die, most of them young children in sub-Saharan Africa[79]. Recently, in March 2005, the Institute de Veille Sanitaire (InVS) warned about an increased risk of chikungunya fever infections in the French territories in the Indian Ocean[262]. By the end of June 2006, the surveillance system estimated that almost 266,000 people (about 35% of the population) had a clinical form of chikungunya and 254 official dead cases were registered on Reunion Island[263]. The main vector of the virus, the tiger mosquito *Aedes albopictus*, was found in several metropolitan districts over the past few years, especially along the Mediterranean coast and in Corsica, prefiguring an increased risk of chikungunya outbreaks in these areas in the future[264].

Prevention and control rely on reducing the number of infected people. A personal protection using repellent products is necessary to minimize the risk of infection and reduces the discomfort caused by mosquitoes. Repellents can be applied to exposed skin or to clothing in strict accordance with product label instructions. Commercial repellents often contain DEET (*N,N*-diethyl-*m*-methylbenzamide), IR3535 (3-[N-acetyl-N-butyl]-aminopropionic acid ethyl ester), Icaridin (1-piperidinecarboxylic acid, 2-(2-hydroxyethyl)-1-methylpropylester) or PMD (*para*-Menthane-3,8-diol)[80-82].

Official authorities are more and more concerned on local development and encourage companies to use natural resources coming from their own region. In addition, a large opinion poll revealed that the population of Corsica would rather apply a repellent product against mosquitoes made for local resources[264-267] with a comparable level of protection than a synthetic repellent product.

In this paper, 19 essential oils from Corsica were screened as a potential active substance in a spatial repellent formulation. Most of the active substances currently available on the market show a “contact” repellent effect; the mosquito approaches the treated skin surface, touches down, but immediately takes off again after a short contact, without biting the person[268]. In the last years, research was also focusing on so called “space”

repellents, which may provide spatial protection – a barrier around the person that inhibits any contact with host-seeking mosquitoes.

Nineteen organic cultivated oils were tested in a bio-assay using a Y-Tube Olfactometer device and *Aedes aegypti* mosquitoes[269]. The y-tube olfactometer allows a quick and efficient screening of new, potential repellent candidates. Two different parameters were considered: the reduction of the mosquito activity inside the olfactometer, due to the presence of an essential oil, and the reduction of the attractivity of a human finger put inside a stimulus chamber behind a filter paper impregnated with an essential oil. In order to evaluate the new method, DEET and PMD were used as reference substances: both are reputed to have a contact repellent effect without any space repellent properties[270].

Additionally, olfactory studies were carried out on human volunteers. The tested corsican oils may be used either as a main active substance to repel mosquitoes (according to the previous tests) or as an additional fragrance in a repellent formulation. The focus was laid on the perception of the olfactory properties of the oils. A selection was performed in a hedonic dimension[271]. The hedonic test describes the preferences of the participants for the selected oils based on the olfactory aspect. The second test was divided into two parts. First, the test persons had to score the oils that were filled in vials, and second the same experiment was repeated with one drop of a solution of the oil directly on the skin. The odor perception of samples from a vial or directly from the skin appears to be different due to the olfactory capabilities of the volunteers.

A well-known and widely used odor for repellent products is *Citronellal*. This molecule was then used as a control standard. Furthermore, females are expected to have a higher olfactory capabilities level than males. This report also focuses on the gender variable and involved 25 males and 25 females as volunteers in the olfactory experiment. Finally, thermogravimetric analyses (TGA) were performed on *Calamintha nepeta*, *Laurus nobilis*, *Rosmarinus officinalis* to determine the weight loss of selected oils at 33°C versus time to provide information on the evaporation rate of the oils.

10.3. Experimental procedures

10.3.1. Material / Equipment

Products 19 Corsican essentials oils from organic farming were provided by Essences Naturelles Corses E.A.R.L (San Nicolao, Corsica Island, France). These oils, extracted by hydrodistillation, were obtained from different parts of the plants: leaves, flowers, branches and resins. Table 10.1 provides a list of oils used in all experiments. Additionally, to these 19 essential oils, citronellal (Merck, Germany; grade: 96%) and 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol (*para*-Menthane-3,8-diol / PMD; Taksago, France), were used as standard controls for the olfactory study. All oils as well as the standard controls were dissolved in absolute ethanol (EtOH; J.T. Baker Netherlands; grade: 99.9%). The solutions were prepared in 10 ml glass vials and for each olfactory test new solutions were used.

Thermogravimetric analysis Essential oils residue samples of $\sim$ 13 mg were subjected to thermogravimetric analysis in a nitrogen atmosphere. A TGA7 from Perkin Elmer corp. (Norwalk, CT) was used. An isotherm heating program was set up at 33°C. This value corresponds to the mean temperature of human skin on the forearm. In order to obtain a low-noise TG signal, a constant gas flow of 70 ml min^{-1} was set for all tests. The precision of temperature measurement for the thermobalance is $\pm$ 1°C. The continuous records of weight loss and temperature were obtained and used to determine the evaporation rates (weight-loss % min^{-1}) of selected oils.

Nr.		Essential Oil	Extracted from	Main components
1	22	*Cistus monspeliensis L.*	Leaves	
2	23	*Lavendula stoechas*	Flowers and panicles	camphor, fenchone, limonen, 1,8-cineole, camphene
3	24	*Eucalyptus globulus*	Leaves	1,8-cineole, α-pinene, α-terpineol, globulol
4	25	*Calamintha nepeta*	Leaves and bloomer herbs	menthone, pulegone, piperitone, limonene
5	26	*Helichrysum italicum*	Leaves	neryl acetate, neryl propionate, 2,4,6,9-tetramethyldec-8-en-3,5-
6	27	*Foeniculum vulgare*	Seeds	(E)-anethole, fenchone, methyl chavicol
7	30	*Myrtus communis L.*	Flowers and leaves	1,8-cineole,α-pinene, limonene
8	31	*Eucalyptus globulus*	Adult leaves	1,8-cineole, α-pinene, aromadendrene
9	32	*Eucalyptus globulus*	Young leaves	1,8-cineole, α-pinene, α-terpineol, globulol
10	33	*Eucalyptus camaldulensis*	Leaves	α-pinene, β-pinene, α-phellandrene, 1,8-cineole
11	34	*Laurus nobilis*	Young growths and leaves	1,8-cineole, α-terpenyl acetate, β-pinene, linalool
12	35	*Cupressus sempervirens*	Branches	α-pinene, α-terpinene, α-terpinolene
13	36	*Inula graveolens*	Leaves	bornyle acetate, p-mentha-1,(7),2-dien-8-ol,τ-cardinol
14	37	*Citrus clementina*	Young branches with small fruits	β-pinene, linalool, limonene
15	38	*Thymus vulgaris*	Flowers	Thymol, β-pinene, α-pinene, carvacol
16	39	*Pistacia lentiscus*	Resins	α-pinene, myrcene, α-phellandrene
17	40	*Helichrysum italicum*, 1 %	Flowers	neryl acetate, neryl propionate, 2,4,6,9-tetramethyldec-8-en-3,5-dione
18	42	*Cistus ladanifer*	Resins	α-pinene, viridiflorol, 2,2,6-trimethylcyclohexanone, verbenone
19	51	Rosmarinus officinalis		α-pinene, verbenone, camphor, bornyl acetate, borneol

Table 10.1: Essential oils used for the repellent experiment and the olfactory study. The oils were all extracted by hydrodistillation in Corsica.

10.3.2. Mosquito repellent studies

Insects Female *Aedes aegypti* from cultures of the Centre for Plant Research of the BAYER AG in Monheim (Germany) were used for the experiments. The mosquitoes were bred under standard conditions at a temperature of 27°C, a relative humidity of 60 – 80 % and a 12:12 hour photoperiod. The light period (150 Lux) was set from 8:00 to 20:00. After hatching of the eggs, larvae were kept in a water basin (30 x 30 x 10 cm) filled with a 1:1 mixture of tap- and deionized water and fed with fish food flakes (Tetra Min®). Before hatching the pupae were transferred to a cage (40 x 30 x 20 cm) and provided with sugar solution (10% dextrose).

Y-Olfactometer Figure 10.1 is a schematic drawing of the Y-tube olfactometer, an improvement of an earlier apparatus to measure the attraction of mosquitoes to volatile stimuli in choice experiments. One olfactometer consists of a 50 cm long Plexiglas® tube, connected to a decision chamber as well as a test and control branch.

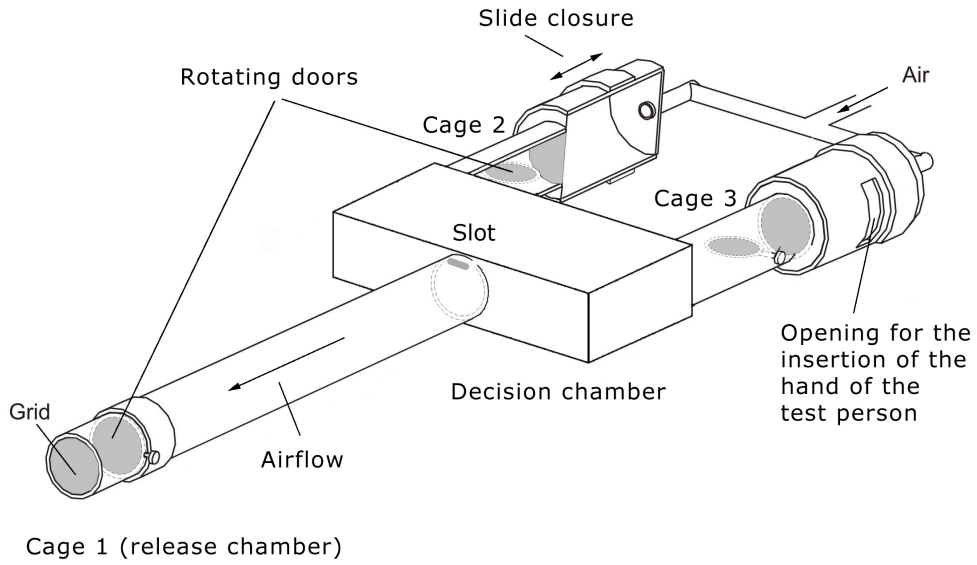


Figure 10.1: Schematic drawing of the Y-tube olfactometer. Cage 1 (start chamber): release chamber for the mosquitoes. Decision chamber: box, in which the mosquitoes can decide between the two branches of the olfactometer. Cage 2 & 3: destination cages. Grid & Rotating doors made from a mesh gauze. Total length of the apparatus ca. 1 m.

At the front end of the apparatus, a release chamber can be connected that is filled with 15 - 20 mosquitoes. Test stimuli can be introduced into the apparatus through the stimulus chambers behind cages 2 and 3. The stimulus chambers consist of metal and were lined with PVC®. A constant air stream of 1120 l/min from a pressurized air system was purified with activated charcoal, then heated and humidified (27± 2°C, 68 ± 5%) before being passed through the olfactometer. The olfactometer was modified in order to measure the space repellent activity of the oils. The Plexiglas® tube connected to the release chamber and the decision chamber was extended by a 6 cm - Plexiglas® tube with a 2.5 cm wide slot, through which a filter paper with a diameter of 2 cm could be introduced into the olfactometer attached to a metal wire. Four identical olfactometers were used for the experiments and were lighted up by two light bulbs (60 W, 280 lux, distance of 55 cm to the olfactometer).

Procedure The olfactometer was operated under standard conditions of temperature and humidity as previously mentioned. For each olfactometer, cohorts of 15-20 female mosquitoes were used and lured out of their breeding cages into the release chambers by a natural stimulus (human hand) in order to ensure that only blood-hungry females were

used for the repellent test. After an acclimatization time of 30 minutes, the first stimulus was tested. During the acclimatization phase, the operator prepared the filter papers with a diameter of 20 mm provided from MELITTA® (Classic 1, Ø 94 mm). The oils were dissolved in ethanol at different concentrations. The filter papers were spread with 0.03 ml of a prepared solution using a graduated glass-pipette (0.1 ml). The operator attached the humidified filter paper to a metal wire and introduced it into the now 56 cm long Plexiglas® tube between the release and the decision chamber of the olfactometer. Immediately afterwards, the finger was held into the opening of the stimuli chamber. The rotating door of the release chamber was opened and mosquitoes were free to enter the apparatus. After 30 seconds, all rotating doors were closed and the filter paper was taken out of the tube.

For the statistical analysis, two behavioral responses of the mosquitoes were recorded: (i) the percentage of mosquitoes found outside the release chamber after 30 s, which gives an indication on the mosquitoes' activity. (ii) The percentage of mosquitoes trapped at the upwind end of the test and control chambers, which respectively provides data of the attractiveness of the natural stimuli (finger) and the control chamber (no stimuli). After each test, the mosquitoes were lured back into the release chamber by reversing the airflow in the olfactometer and using the hand as a natural attractant. Each stimulus was tested 8 times with an interval of 15 minutes between each subsequent test. A permutation scheme was used to avoid an adaptation of the mosquitoes to the different test stimuli.

10.3.3. Human Olfactory study

Volunteers The experiment was conducted at the Institute of Physical and Theoretical Chemistry at the University of Regensburg. A total of 25 males and 25 females from different working groups participated in the study. Their age ranged from 22 to 56 with a mean of 25 years. All subjects were in a good state of health and showed no signs of head cold or allergy.

Hedonic test Each participant was presented with 21 solutions. The samples were labeled and presented in random order. Each individual was asked to smell the 21 solutions out of the vials starting with the first five samples, followed by a 20 minutes

break outside the test room. In total, the olfactory experiment lasted 2 hours for each participant. The hedonic test was a first screening to assess the preference of the volunteers for the oils. The hedonic test scored each sample using a default scale from -3 to +3 (-3 corresponds to a very unpleasant odor, -2 unpleasant odor, -1 slightly unpleasant, 0 neutral, +1 slightly pleasant, +2 pleasant and +3 a very pleasant odor). Female and male total preferences for each solution were combined and the means were calculated. The same calculations were done by gender.

Acceptance test The same procedure as previously described was used for the acceptance study. This test was divided into two parts, consisting on the tolerance of the different odors emanated from the solutions as a main fragrance in a specific repellent product. As an example, the majority of the volunteers would have scored a coffee bean with values between +2 and +3 on the hedonic dimension but would probably not accept to use a product that emits a coffee odor and repels mosquitoes. First, the participants smelled each solution directly out of the glass vial. Secondly, a one drop solution was applied to the skin and left to dry for a few seconds before the evaluation. The forearm between wrist and elbow was used as a test area. Prior to the experiment, the skin was washed with fragrance-free soap and 50% isopropanol and then dried with a paper towel. During this second session, the participants were asked to score each solution for both tests using another point scale from -2 to +2 (- 2 corresponds to absolutely not suitable as a repellent product, -1 not suitable, 0 neutral, +1 suitable, + 2 very well suitable as a repellent product). Same calculations procedures set up for the hedonic part were performed for the 19 essential oils and the two control molecules.

10.3.4. Statistical analysis

For the analysis of all collected data, the statistical software package SPSS^{TM} 7.5 was used. Significance tests were carried out using one-way analysis of variance (ANOVA) and T-test ($P<0.05$). As citronellal is widely used in repellent products as a main odor, statistics were based on this molecule to relate every oil to a significant score ($P<0.05$).

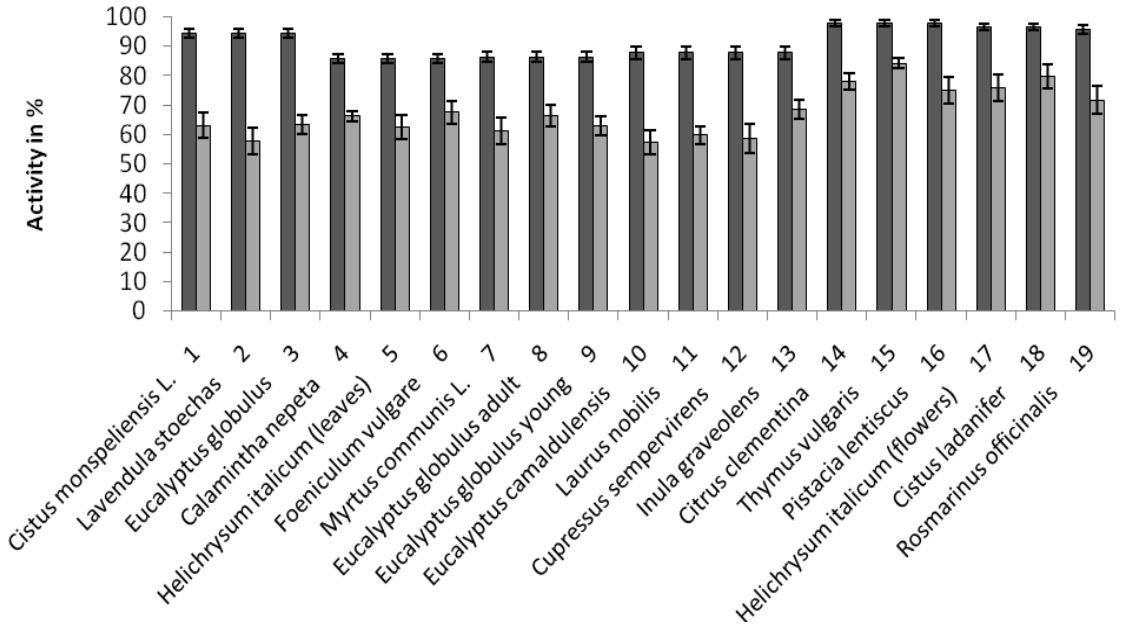
10.4. Results and discussion

10.4.1. Mosquito repellent study

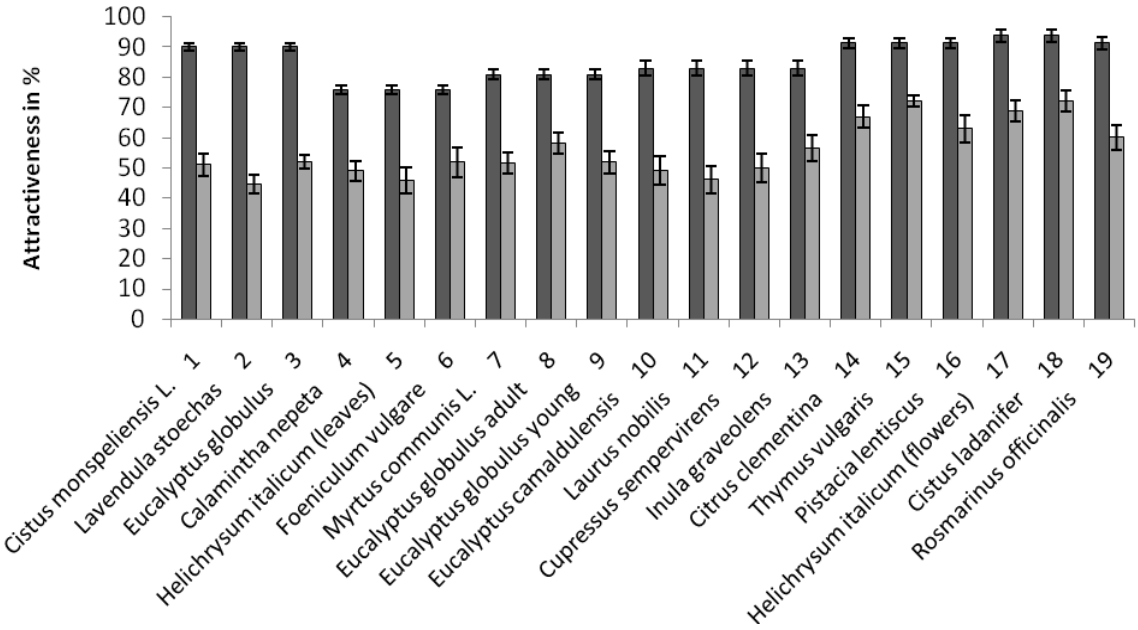
One part of this project was the evaluation of 19 Corsican oils as potential "space" repellents. A first screening was set up regarding the activity of the mosquitoes using two stimuli: the finger as a standard control and the oils as tests stimulus. The finger was placed into the stimuli chamber whereas the impregnated filter paper was introduced into the slot of the modified Plexiglas® tube. Graph. 10.1 exhibits the reduction of the mosquito activity, while an essential oil is tested in the olfactometer. Regarding the standard control (finger), the activity values varied from 85% to 97%, which is in the relative mean according to previous experiments in the past. The activity depends on the age of the mosquitoes, the air pressure, the temperature and the humidity in the lab. These climatic parameters undergo minor day-to-day variations and may influence the physical fitness of the mosquitoes.

In comparison to the finger as a single stimulus, all oils caused a reduced activity of the test mosquitoes in the olfactometer. The values varied from 57% for *Eucalyptus camaldulensis* to 84% for *Thymus vulgaris*. A total of 4 essential oils yielded promising results with an activity level of around 60%: *Lavendula stoechas*, *Helichrysum italicum* (leaves), *Laurus nobilis* and *Cupressus sempervirens*. ANOVA analysis showed a significant reduction of activity between these 4 oils and the finger ($p<0.05$, Tukey´s HSD-Test): while the oils were tested, a mean reduction of 30% was observed. In the presented cases, this means that more mosquitoes remained in the release chamber instead of entering the tube and trying to bite the human finger.

A similar experiment was set in order to screen the attraction of the test mosquito population towards the finger. The same method was used, but this time, the percentage of mosquitoes trapped at the upwind end of the test chamber, where the finger was introduced into the apparatus, was calculated. This test gives indications on the ability of an essential oil to disturb the up-wind flight of the test mosquito. Graph. 10.2 shows the level of attraction of the finger, while 19 Corsican oils were tested.



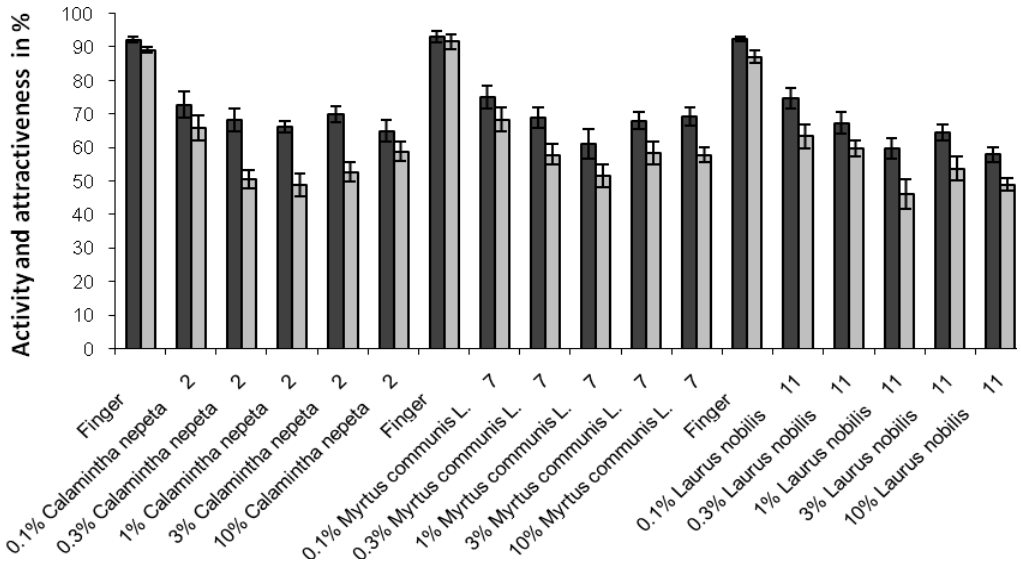
Graph. 10.1: Activity test of 19 Corsican essential oils with *Aedes Aegypti* mosquitoes. ■ corresponds to the mean number of mosquitoes (in %, the standard error is in bars) that left the release chamber, when a finger was used as a control stimulus. ■ is the mean number of active mosquito (in %, the standard error is in bars), when the finger and the essential oil were tested. Number of repetitions: n=8.



Graph. 10.2: Attractiveness test on the mosquitoes *Aedes aegypti* with 19 Corsican essential oils. ■ corresponds to mean number of attracted mosquito (in %, standard error is in bars) trapped in the stimulus chamber using the finger as a control stimulus, ■ corresponds to mean number of attracted mosquito (in %, standard error is in bars) trapped in the stimulus chamber using the finger and the essential oil. Number of repetitions: n=8.

As previously, the values of the standard control (finger) reflect the fluctuation of the physical activity of the mosquitoes. The attractivity of the finger ranges from 76% to 94%. When an essential oil was tested, the attractiveness of the finger varied from 45% for *Lavendula stoechas* to 72% for *Thymus vulgaris*. Each oil tested caused a reduction of the attractiveness of the finger with promising scores for 6 essential oils: *Cistus monspeliensis L.*, *Calamintha nepeta*, *Helichrysum Italicum* (leaves), *Eucalyptus camaldulensis*, *Laurus nobilis*, *Cupressus sempervirens*. Compared to tests of the finger alone, the mean reduction of the attractiveness was 35%, which is similar to reduction of activity observed during the previous tests. The activity of the mosquitoes and their attraction towards the finger are linked together: if the mosquito leaves the release chamber and is not disturb by the presence of an essential oil, it is immediately attracted by the finger and no disturbance of the mosquito can be noticed. This observation demonstrates that there is no loss of the sensory activity due to the presence of the oil.
5 essential oils displayed promising scores on both criteria, the reduction of the flight activity and the reduction of the attractiviy of the finger: *Eucalyptus camaldulensis*, *Lavendula stoechas*, *Helichrysum Italicum* (leaves), *Laurus nobilis*, *Cupressus sempervirens*. Dosage curves with different concentrations of these oils were considered, in order to investigate, if the observed reduction levels caused by these oils can be increased.

Previous experiments were based on a 1% oil solution in ethanol. Four other concentrations at 0.1%, 0.3%, 3%, 10% in ethanol were tested from the following selection of essential oils: *Calamintha nepeta*, *Helichrysum Italicum* (leaves) and *Laurus nobilis*. Graph. 10.3 contains the results of the mosquito activity and the attractiveness of the finger, while different concentrations of the oils were tested.

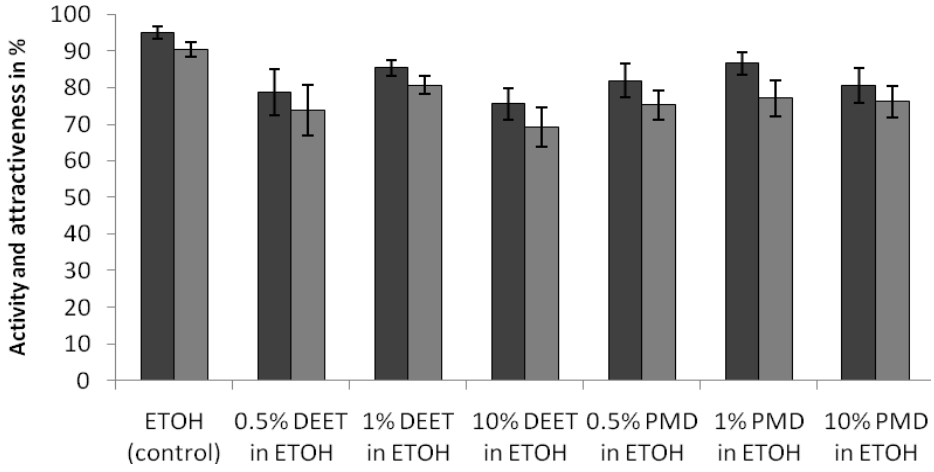


Graph. 10.3: Concentration-dependent olfactometer test of the activity and the attractiveness of *Aedes aegypti* with three selected oils: *Calamintha nepeta*, *Myrtus communis L.* and *Laurus nobilis*.

corresponds to the mean number of active mosquitoes (in %, sta ndard error is in bars) that left the release chamber and flew up-wind, is the mean number of attracted mosquito (in %, the standard error is in bars), that were found inside the test chamber, where the finger was introduced. Number of repetitions: n=8.

The results of this section show that an increase of the concentration of the three selected oils did not lead to a stronger effect on the activity of the test mosquitoes or the attractivity of the finger. The used concentrations, between 0.1% and 10%, did not show any significant differences in the mean number of active mosqutioes and the mean number of mosquitoes found inside the test chamber, where the finger was introduced ($p>0.05$, Tukey´s HSD-Test). It is possible that low concentration of essential oil already rapidly saturates the mosquitoes' sensors so that high concentrations of essential oils do not extend the repellent properties.

The last experiment was performed with two repellent molecules well-known to exhibit contact repellency instead of a space repellent effect. DEET and PMD were both tested at different concentrations. Results are presented on Graph. 10.4.



Graph. 10.4: Concentration-dependent y-tube olfactometer test on the activity of *Aedes aegypti* and the attractiveness of a human finger with DEET and PMD.

corresponds to the mean number of active mosquitoes (in %, the standard error is in bars), is the mean number of attracted mosquito (in %, the standard error is in bars). Ethanol was used as a control. Number of repetitions: n=8

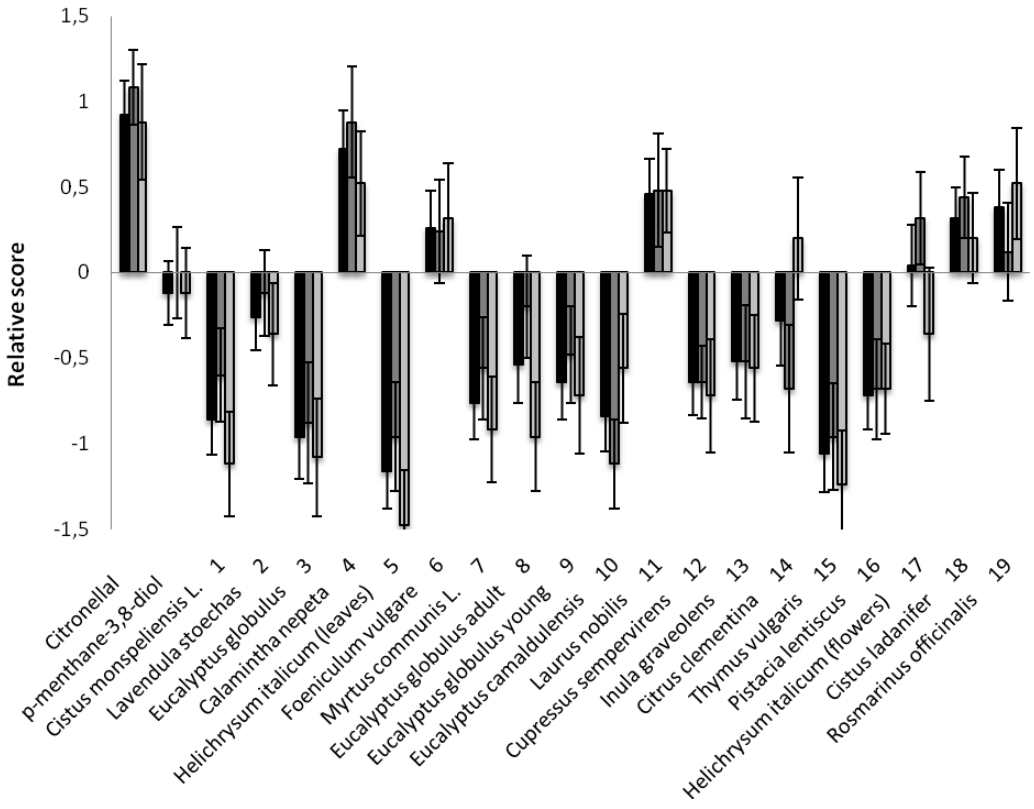
The results did not show any space repellent properties of DEET and PMD on *Aedes aegypti* at the tested concentrations. A reduction of the test mosquito's activity and attractivity of the finger is observed, without any significant differences according to ANOVA's calculations between the test molecules and the control (ethanol + finger). Previous studies with PMD and DEET using "arm cage" tests confirmed this result. This experiment was performed in order to compare the 19 essentials, which may have space repellent properties and two control substances, which exhibit contact properties. For instance, 1% *Laurus nobilis* oil in alcohol yielded an activity reduction of 30%, the attractivity of the finger was reduced by 40 %. When DEET was used at the same concentration, only a 10% reduction of the flight activity and a 11% reduction of the attraction of the finger could be observed. These results indicate that the selected Corsican oils have a stronger spatial impact on the test mosquitoes than DEET and PMD.

Finally, three essential oils were found to be most promising, concerning their spatial repellent properties: *Lavendula stoechas*, *Helichrysum italicum* (leaves) and *Laurus nobilis*. A certain reduction of the attractivity of the finger is obtained when these oils are placed in the olfactometer.

10.4.2. Hedonic dimension

A first Hedonic test was carried out on 50 volunteers with the 19 essential oils and the two controls. *para*-Menthane-3,8-diol has a faint mint odour hardly detectable by hunan noises while citronellal has a strong lemon scent. PMD was chosen for this study because it showed in the past great properties to repel mosquitoes[93,272] and might be used as main active in a repellent product. Besides, this molecule is natural, extracted from the leaves of *Eucalyptus Citriodora* or synthesized from L-citronellal[75,76]. Citronellal was also used as a control standard. Consumers could be conditioned to the lemon odor for a repellent product even if citronellal shows a short-lasting protection on mosquitoes.

When all the female and male volunteers' preference totals for each sample were combined, the mean score was calculated. Male and female results were treated similarly and the overall result of this study is shown in Graph. 10.5.



Graph. 10.5: Hedonic test on 50 Human volunteers 50 Human volunteers (25 males, 25 females) with 19 oils and two standard molecules, citronelall and PMD.

corresponds to the average value for both genders, male preference, female preference.

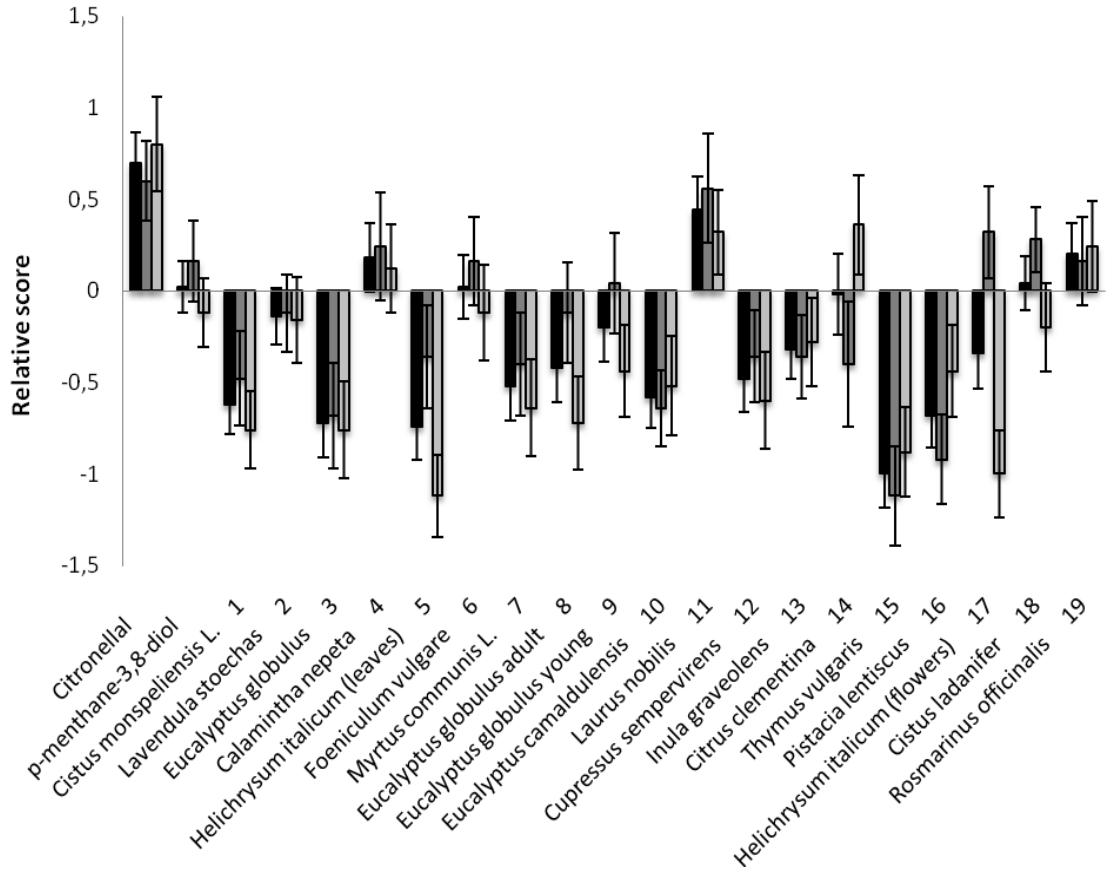
It appears that male and female preferences are, in general, almost identical as both gender types showed greatest liking for the oils *Calamintha nepeta*, *Foeniculum vulgare*, *Laurus nobilis*, *Citrus clementina*, *Helichrysum italicum* (flowers), *Cistus ladanifer*, *Rosmarinus officinalis*. The only difference of opinion was on the oil *Citrus clementina*. This oil was greatly rejected by males. Clementine offers a fresh, crisp citrus aroma, which may displease male due to its fruity and strong odor. Previous studies showed that men usually prefer classical and light odors, while women have a greater sense of adventure and have a larger sensory panel[273,274]. As expected, the results with citronellal on both genders were superior and the sample was by far the most liked aroma on this hedonic study. PMD's score indicates that this compound has barely none significant preference for any gender and even a slight rejection for female. It was noted that the order of the scored oils do not contribute to any selection.

At the end of this hedonic study, 6 oils were preselected: *Calamintha nepeta, Foeniculum vulgare, Laurus nobilis, Helichrysum italicum (flowers), Cistus ladanifer, Rosmarinus officinalis.*

10.4.3. Acceptance study

The two experiments reported here present the acceptance of these 19 essential oils to be integrated in a repellent product as a minor or main fragrance. It has been previously reported that citronellal was a common aroma used in several repellents and consumers would rather buy a product with a lemon odor. ANOVAs conducted on the acceptance score of these oils filled in a vial as well as on the skin revealed differences between male and female volunteers. Each oil was tested and compared to the standard citronellal. The oils were rejected each time the T-test ($P<0.05$) showed on the score a significant difference with the standard.

The first test was performed out a vial. Graph. 10.6 shows the slight difference between the two genders in term of acceptance of these oils with the exception of 5 oils: *Foeniculum vulgare*, *Eucalyptus globulus* (young leaves), *cistrus clementina*, *Helichrysum italicum (flowers), Cistus ladanifer*. These 5 essential oils were rejected from the study as long as they are not accepted by both genders.



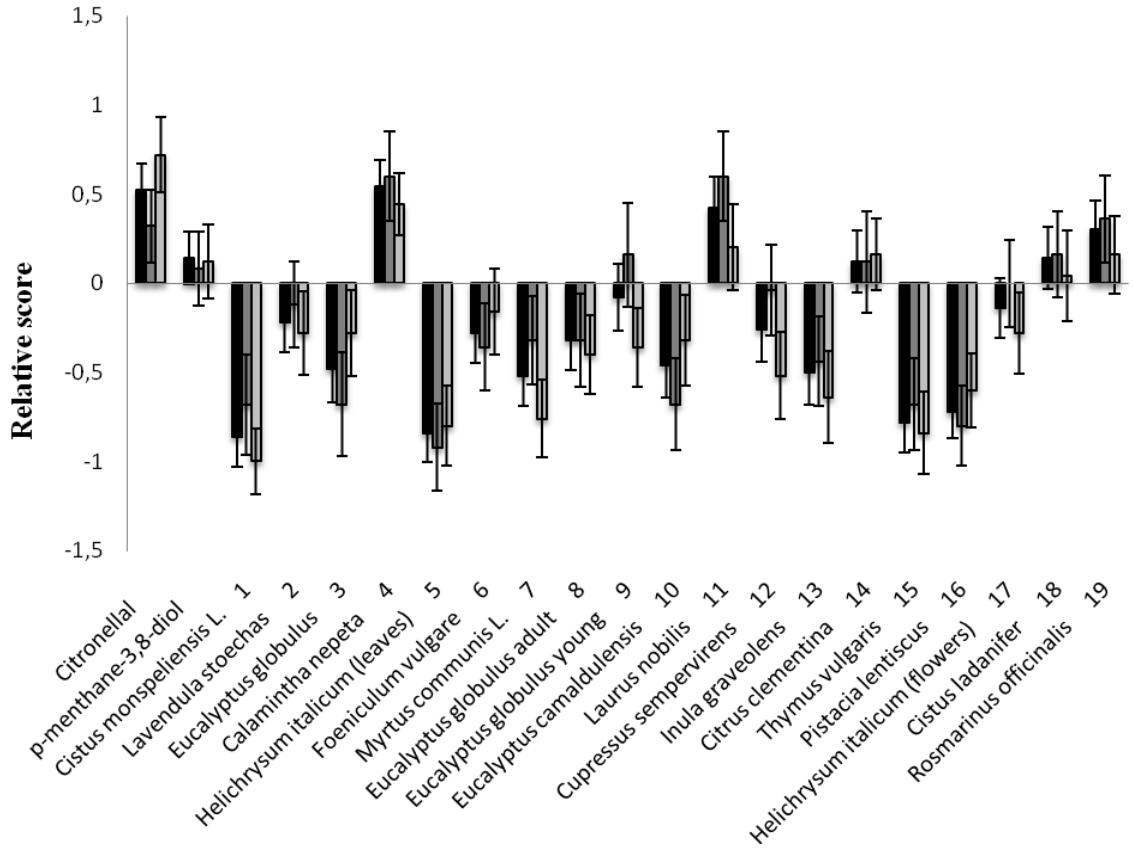
Graph. 10.6: Acceptance test for a repellent product on 50 Human volunteers (25 males, 25 females) with 19 oils and two standard molecules out of a vial.

corresponds to the average value of both genders, male acceptance, female acceptance.

The statistical analysis on the male gender showed a great difference between citronellal and 2 oils (*Thymus pulgaris* and *Pistacia lentiscus*). Theses samples were automatically rejected. Another selection by the negative scores completed the study. Finally, for the male gender, 7 oils were selected: *Calamintha nepeta, Foeniculum vulgare, Eucalyptus globulus* (young leaves), *Laurus nobilis, Helichrysum italicum (flowers), Cistus ladanifer, Rosmarinus officinalis.* The same process was done with the female gender and a selection of 4 oils was set (*Calamintha nepeta*, *Laurus nobilis*, *Citrus clementina*, *Rosmarinus officinalis*). The final selection was based on the acceptance of the oils from both genders. *Citrus clementina* is for instance rejected by male on the hedonic and the acceptance test and then is put aside. Same conclusion for *Helichrysum italicum* (flowers), *Eucalyptus globulus* (young leaves) and *Foeniculum vulgare* oils, which are rejected by female.

At the end 3 essentials oils remained as a potential fragrance for a repellent product: *Calamintha nepeta, Laurus nobilis, Rosmarinus officinalis*. This study indicates that gender has a little influence on olfactory acuity but generally an individual selection is done without giving any conclusions or relative explanations on the differences observed. There again the citronellal sample showed the highest score for both genders and demonstrated the relative preference on the panel for this lemon odor for a repellent product. PMD for instance, which has a very low vapor pressure is not approved for this specific application and is even rejected by females.

The second part of this acceptance test was performed on the participants' skin. Following the process previously described on the material and method part, volunteers were invited to put one drop of the solutions on their skin and then smell. Usually the odor perception out of a flacon or any king of support is slightly different that it is on on a human skin. Graph. 10.7 accounts the results for this acceptance study.

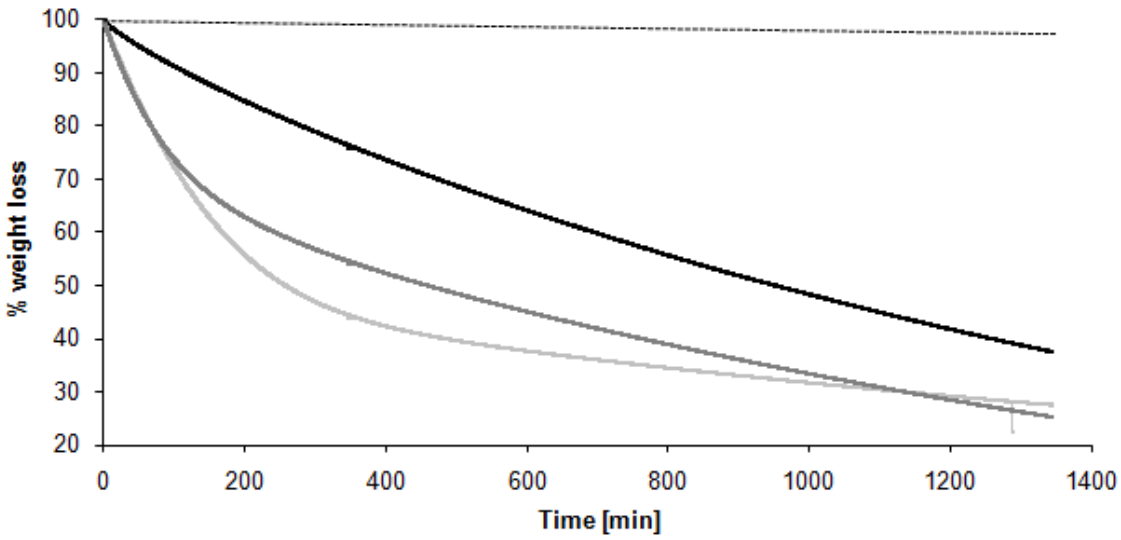


Graph. 10.7: Acceptance test for a repellent product on 50 Human volunteers (25 males, 25 females) with 19 oils and two control molecules on the skin.
corresponds to the average value of both genders, male acceptance, female acceptance.

As expected, the present study shows nearly no differences between both genders excepted for one oil: *Eucalyptus globulus* young. This oil was rejected on the previous work. The same selection framework was carried out combining the hedonic test and the acceptance one out of the vial to reject the selected oils. At the end, 3 essential oils out of 19 remained: *Calamintha nepeta, Laurus nobilis, Rosmarinus officinalis*. These oils presented more or less the same scores and were not significantly different from citronellal. Additionally, citronellal was over again the favor aroma of the panel and confirmed its high acceptance for a repellent product. PMD was well accepted on the skin due probably to its fresh and smooth odor.

10.4.4. Thermogravimetric analysis

Additionally, to the repellent study and the olfactory tests, thermogravimetric analysis was performed on 3 selected oils: *Calamintha nepeta, Laurus nobilis, Rosmarinus officinalis*. The TGA shows the stability of the oils as a function of time at 33°C (skin temperature). *para*-Menthane-3,8-diol (PMD) was also used as a reference molecule. The evaporation curves are represented in Graph. 10.8.



Graph. 10.8: TGA curves of the evaporation rate of *Calamintha nepeta* (—), *Rosmarinus officinalis* (—), *Laurus nobilis* (—) and p-menthane-3,8-diol (····) under a nitrogen atmosphere at 33°C ± 1 °C.

Calaminhta nepeta is the most stable with 61% weight loss at 33°C over 24 hours. The two others have a similar behavior with around 73% weight loss after 1 day. The TGA exhibit differences between these oils due mainly to their composition. It's well known that terpens (hydrocarbons) have a great vapor pressure compare to others structures. *Rosmarinus officinalis* and *Laurus nobilis* are composed respectively of 27% and 40% of terpenes while *Calaminhta nepeta* contains only 11%. As expected *Calimintha nepeta* has the slowest evaporation rate as a comparison to the others and may ideally be used in a repellent product for a longer effect. However, the reference molecule PMD has a very slow evaporation as shown on the figure. After 24 hours test, only 3 wt% loss was found, which is very low compare to these three oils. The long-lasting repellent property displays by the PMD is partly due to its slow evaporation rate over time and also certainly from its relatively low penetration action through the stratum corneum (layer of the epidermis). In consequence, regarding the results obtained from these three oils, the evaporation rate is significantly different compare to the PMD molecule and a long-lasting protection by using these oils might not be reached. They may lose their repellent properties rapidly over time. Thus, they are mostly used as additional fragrances in repellent products and not as main active substances[88]. The selected Corsican essentials oils would rather be used as promoters for others repellent molecules like DEET or PMD to extend the time protection.

10.5. Conclusion

In this study, 19 essential oils form Corsica Island (France) were tested as potential repellents against *Aedes aegypti*. Space repellent properties were evaluated in a Y-tube olfactometer and three oils displayed a promising effect on the test mosquitoes: *Lavendula stoechas*, *Helichrysum italicum* (leaves) and *Laurus nobilis*. The activity of the test mosquitoes as well as the attractivitiy of the finger were reduced to 30%, while testing these oils and compared to the finger alone as a control stimulus. These results are not sufficient enough to use these oils as main active substances in a mosquito repellent formulation but, they might be employed as a secondary agent to extend the protection duration of more specific repellent molecules like PMD or DEET. Additionally, an olfactory test on Human volunteers was carried out with these 19 essential oils, showing

great differences on a hedonic dimension and on the acceptance of these oils as fragrances for a repellent product. *Calamintha nepeta*, *Laurus nobilis*, *Rosmarinus officinalis* conducted promising scores on both criteria. The participants were prepared to use these three essential oils as a replacement for the classical citronellal odor in a repellent product. Finally, *Laurus nobilis* displayed bright results on both studies (repellency, olfactory), but as shown on the thermogravimetric analysis its evaporation rate may disturb its ability to be used as a main active.

General conclusion

In the present thesis, *para*-Menthane-3,8-diol (First Part) and a newly derived so-called PMD-Succinate (Second Part) were studied as effective repellent actives. PMD is a well-known molecule, which is slightly less efficient than DEET (*N,N*-diethyl-m-methylbenzamide) with certain mosquito's species, but its toxicity is very low. Nevertheless, the use of PMD in repellent formulations involves major problems: a low solubility in aqueous media, a tendency to penetrate rapidly through the upper skin layers and a lower repellent activity. These aspects influence significantly the use of pure PMD as a repellent active in manufactured products.

PMD was successfully synthesised following a green, fast, easy and cost-effective process, from the essential oil of *Eucalyptus citriodora*. With adapted choices of acid, temperature and time reaction, cyclisation of (+)-citronellal from the oil gave *cis* and *trans*-*para*-Menthane-3,8-diol with a high selectivity, as described in Chapter 2. On a bio-assay, this natural PMD in combination with others acetals and terpenes displayed a long-lasting protection (303 minutes) against *Aedes aegypti* mosquitoes. In Chapter 3, a dose test of pure *para*-Mentahne-3,8-diol in isopropanol using *Aedes aegypti* mosquitoes in cage tests was performed to determine the usable concentration of active (20% w/w) for future formulations. PMD was then incorporated in either surfactantless (isopropanol / water) or classical (isopropanol / selected surfactants / water) microemulsions showing for both systems monodisperse aggregates with droplets size at around 13 nm. The objective was to reduce the amount of alcohol in the formulation, which is known to enhance the penetration of the main active through the upper skin layers. For instance, the formulation: 20% w/w PMD, 25% Isopropanol, 2% Cremophor® RH40, 1% 2-ethyl-1,3-hexanediol, 3% Texapon® N70, 3% Ethyl (S)-(-) lactate, 46% Water was 22% more

efficient on mosquitoes (385 min complete protection) by using 34% less alcohol, than a surfactantless microemulsion. This study showed that the choice of the formulation system is a key factor when designing new long-lasting repellent with PMD.

In Chapter 4, the development of repellent formulations using *para*-Menthane-3,8-diol was extended to Oil-in-Water emulsions. With adapted used of emollients, solvents, chelating agents, rheology modifiers, humectants, pH adjusters, preservatives, antioxidants and perfumes, the new O/W formulation reached 480 min complete protection against *Aedes aegypti*, which is statistically comparable to the reference product on the market, the Autan® Protection Plus from SC Johnson. Organoleptic parameters and microbiological studies were also investigated. Again, these results proved that pure PMD is able to achieve the same efficacy as standard repellents composed of KBR 3023 for instance, by developing a suitable long-lasting formulation.

PMD/Cyclodextrins inclusion complexes have been studied in solution and under a solid state (powder) in Chapter *5* in order to develop efficient surfactant and alcohol-free repellent formulations. The complexation and the orientation of PMD with β-CD, HP-β-CD and γ-CD in solution has been examined by ^{1}H NMR and 2D NMR. It was proved that the inclusion complex was formed between PMD and the three CDs with a 1:1 stoichiometry. Solid complexes of PMD with HP-β-CD and γ-CD have also been investigated. A maximum of complexed PMD is reached after 6h stirring at room temperature, with 20% of HP-β-CD in solution and a mass ratio PMD/CD of 1/10. The complex has a high solubility in water (>63.6% w/w), which potentially would be of great interest for repellent formulations. Unfortunately, HP-β-CD:PMD inclusion complex against *Aedes aegypti* did not show any protection activity.

In a second part, a derivative of *para*-Menthane-3,8-diol was developed and synthesized by a suitable esterification using succinic anhydride to produce PMD-Succinate (Chapter 6). The characterisation of the novel molecule was done by elemental analysis, IR, ^{1}H-NMR, ^{13}C-NMR spectra and GC-MS. PMD-S is water-soluble and thus enables the design of repellent formulations without the addition of undesirable additives such as alcohols or surfactants. Repellent studies on *Aedes aegypti* with 20% of PMD-S in water displayed a high repellent activity with 300 minutes of protection, which is statistically comparable to PMD and DEET in alcoholic solutions.

The physical and chemical properties of PMD-Succinate were reported in Chapter 7. Krafft point, pKa, surface activity, solubility, binary phase diagram, hydrolysis and TGA were investigated. As main results, the new repellent molecule acts as a hydrotrope with a MHC of 0.39M, is water-soluble up to 250g/L and has the property to increase the solubility of current repellent actives in water. An example of formulation was given using the system PMD-S/PMD/Water.

In Chapter 8, the cytotoxicity of PMD-Succinate was evaluated in comparison to 4 repellent actives (IR3535, PMD, DEET, KBR 3023) using the MTT bioassay on Hela and SK-Mel -28 cell lines. It can be seen that the skin tolerance of the new active is particularly good with the higher IC_{50} values (lower toxicity), whereas the expected eye irritation is higher than those of current repellent actives, but still lies in the same order of magnitude. As a preliminary result, the use of PMD-S for leave-on repellent formulations is harmless with regard to skin irritations and allergic reactions.

In order to improve the synthesis of PMD-Succinate with eco-friendly considerations, a new process was established in Chapter 9. An easy and fast method was developed by using a conventional mixing and an ion-exchange resin for the separation method that enables one to recycle unused reactive materials such as pure PMD for new runs. The yield of the reaction was 46% higher than with the first method, reaching nearly 60%. This protocol is suitable to produce PMD-Succinate in larger scale production.

Finally, in a third part, repellent studies on mosquitoes and human olfactory tests (hedonic dimension and acceptance study) were explored with 19 essential oils from Corsica (France). *Lavendulas stoechas*, *Helichrysum italicum* and *Laurus nobilis* have a strong spatial repellent impact on mosquitoes. For the hedonic dimension as well as the acceptance study, *Laurus nobili,* was the preferred essential oil. In a near future, this oil may be integrated in repellent formulations as a spatial repellent booster and as a substitution of common citronellal for a fragrance signature.

As a general conclusion, an extensive study on the repellent active *para*-Menthane-3,8-diol has been performed ranging from a novel green synthesis process to the development of different formulation systems to reach long-lasting protection against mosquitoes comparable to marketed products. Due to the poor water solubility of PMD, a new water-soluble derived agent from PMD, so-called PMD-Succinate was developed and

synthesized under an eco-friendly procedure. It has been found that this molecule has a protection action against mosquitoes statistically comparable to PMD or DEET and has the additional property to increase the water solubility of current insect repellent actives. These two advantages make it possible to give a booster effect in terms of protection and also allow one combine several repellent actives in the same aqueous formulation in order to reach a higher repellent effect on different mosquito species.

Bibliography

[1] In *Medical entomology for students*; 4 ed.; Service, M., Ed.; Cambridge University Press: Cambridge, 2008, p 1.

[2] M. S. Fradin. *Ann Intern Med.* 128:931, 1998.

[3] K. O. Murray, E. Mertens, and P. Desprès. *Veterinary Research.* 41:67, 2010.

[4] J. T. Roehrig. *Viruses.* 5:3088, 2013.

[5] B. P. Granwehr, K. M. Lillibridge, S. Higgs, P. W. Mason, J. F. Aronson, G. A. Campbell, and A. D. Barrett. *Lancet Infect Dis.* 4:547, 2004.

[6] M. C. da Costa-Ribeiro, R. Lourenco-de-Oliveira, and A. B. Failloux. *Mem Inst Oswaldo Cruz.* 101:917, 2006.

[7] World Health Organisation. *Yellow fever fact sheets*: http://www.who.int/mediacentre/factsheets/fs100/en/.

[8] J. Boeckh, H. Breer, M. Geier, F.-P. Hoever, B.-W. Krüger, G. Nentwig, and H. Sass. *Pesticide Science.* 48:359, 1996.

[9] M. Ditzen, M. Pellegrino, and L. B. Vosshall. *Science.* 319:1838, 2008.

[10] E. E. Davis. *J Med Entomol.* 22:237, 1985.

[11] S. Bhatt, P. W. Gething, O. J. Brady, J. P. Messina, A. W. Farlow, C. L. Moyes, J. M. Drake, J. S. Brownstein, A. G. Hoen, O. Sankoh, M. F. Myers, D. B. George, T. Jaenisch, G. R. Wint, C. P. Simmons, T. W. Scott, J. J. Farrar, and S. I. Hay. *Nature.* 496:504, 2013.

[12] O. J. Brady, P. W. Gething, S. Bhatt, J. P. Messina, J. S. Brownstein, A. G. Hoen, C. L. Moyes, A. W. Farlow, T. W. Scott, and S. I. Hay. *PLoS Negl Trop Dis.* 6:e1760, 2012.

[13] World Health Organisation. *Dengue fact sheets*: http://www.who.int/mediacentre/factsheets/fs117/en/.

[14] P. Renault, J. L. Solet, D. Sissoko, E. Balleydier, S. Larrieu, L. Filleul, C. Lassalle, J. Thiria, E. Rachou, H. de Valk, D. Ilef, M. Ledrans, I. Quatresous, P. Quenel, and V. Pierre. *Am J Trop Med Hyg.* 77:727, 2007.

[15] E. A. Gould, P. Gallian, X. de Lamballerie, and R. N. Charrel. *Clinical Microbiology and Infection.* 16:1702.

[16] M. C. Jaffar-Bandjee, D. Ramful, B. A. Gauzere, J. J. Hoarau, P. Krejbich-Trotot, S. Robin, A. Ribera, J. Selambarom, and P. Gasque. *Expert Rev Anti Infect Ther.* 8:987, 2010.

[17] M. Sourisseau, C. Schilte, N. Casartelli, C. Trouillet, F. Guivel-Benhassine, D. Rudnicka, N. Sol-Foulon, K. Le Roux, M. C. Prevost, H. Fsihi, M. P. Frenkiel, F. Blanchet, P. V. Afonso, P. E. Ceccaldi, S. Ozden, A. Gessain, I. Schuffenecker, B. Verhasselt, A. Zamborlini, A. Saib, F. A. Rey, F. Arenzana-Seisdedos, P. Despres, A. Michault, M. L. Albert, and O. Schwartz. *PLoS Pathog.* 3:e89, 2007.

[18] G. Borgherini, P. Poubeau, A. Jossaume, A. Gouix, L. Cotte, A. Michault, C. Arvin-Berod, and F. Paganin. *Clin Infect Dis.* 47:469, 2008.

[19] M. Service. *Medical entomology for students*, 4 ed. Cambridge University Press: Cambridge, 2008.

[20] P. J. McCall, and G. Eaton. *Med Vet Entomol.* 15:197, 2001.

[21] J. D. Edman, and H. C. Lynn. *Entomologia Experimentalis et Applicata.* 18:492, 1975.

[22] A. E. Eiras, and P. C. Jepson. *Bulletin of Entomological Research.* 84:207, 2009.

[23] S. B. McIver. *J Med Entomol.* 19:489, 1982.

[24] W. Takken. *Insect Science and its Application.* 12:287, 1991.

[25] W. Takken, and B. G. Knols. *Annu Rev Entomol.* 44:131, 1999.

[26] J. Bohbot, R. J. Pitts, H. W. Kwon, M. Rutzler, H. M. Robertson, and L. J. Zwiebel. *Insect Mol Biol.* 16:525, 2007.

[27] L. J. Zwiebel, and W. Takken. *Insect Biochem Mol Biol.* 34:645, 2004.

[28] A. E. Eiras, and P. C. Jepson. *Bulletin of Entomological Research.* 81:151, 2009.

[29] W. Takken, and D. L. Kline. *J Am Mosq Control Assoc.* 5:311, 1989.

[30] C. Costantini, G. Gibson, N. Sagnon, A. Della Torre, J. Brady, and M. Coluzzi. *Med Vet Entomol.* 10:220, 1996.

[31] C. Costantini, N. F. Sagnon, A. della Torre, M. Diallo, J. Brady, G. Gibson, and M. Coluzzi. *Am J Trop Med Hyg.* 58:56, 1998.

[32] H. I. Maibach, W. A. Skinner, W. G. Strauss, and A. A. Khan. *JAMA.* 196:263, 1966.

[33] M. S. Mayer, and J. D. James. *Bulletin of Entomological Research.* 58:629, 2009.

[34] C. E. Schreck, D. L. Kline, and D. A. Carlson. *J Am Mosq Control Assoc.* 6:406, 1990.

[35] U. R. Bernier, D. L. Kline, D. R. Barnard, C. E. Schreck, and R. A. Yost. *Anal Chem.* 72:747, 2000.

[36] U. R. Bernier, D. L. Kline, C. E. Schreck, R. A. Yost, and D. R. Barnard. *J Am Mosq Control Assoc.* 18:186, 2002.

[37] M. Geier, H. Sass, and J. Boeckh. *Ciba Found Symp.* 200:132, 1996.

[38] J. G. Logan, M. A. Birkett, S. J. Clark, S. Powers, N. J. Seal, L. J. Wadhams, A. J. Mordue Luntz, and J. A. Pickett. *J Chem Ecol.* 34:308, 2008.

[39] Y. T. Qiu, R. C. Smallegange, J. J. Van Loon, C. J. Ter Braak, and W. Takken. *Med Vet Entomol.* 20:280, 2006.

[40] C. R. Williams, S. A. Ritchie, R. C. Russell, A. E. Eiras, D. L. Kline, and M. Geier. *J Chem Ecol.* 32:1625, 2006.

[41] A. C. Morrison, E. Zielinski-Gutierrez, T. W. Scott, and R. Rosenberg. *PLoS Medicine.* 5:e68, 2008.

[42] World Health Organization. *Safety of pyrethroids for public health use*: http://www.who.int/whopes/resources/who_cds_whopes_gcdpp_2005.10/en/.

[43] T. Chareonviriyaphap, A. Prabaripai, and M. J. Bangs. *J Am Mosq Control Assoc.* 20:45, 2004.

[44] J. P. Grieco, N. L. Achee, T. Chareonviriyaphap, W. Suwonkerd, K. Chauhan, M. R. Sardelis, and D. R. Roberts. *PLoS One.* 2:e716, 2007.

[45] P. Mongkalangoon, J. P. Grieco, N. L. Achee, W. Suwonkerd, and T. Chareonviriyaphap. *J Vector Ecol.* 34:217, 2009.

[46] J. Hemingway, N. J. Hawkes, L. McCarroll, and H. Ranson. *Insect Biochem Mol Biol.* 34:653, 2004.

[47] T. E. Erlanger, J. Keiser, and J. Utzinger. *Med Vet Entomol.* 22:203, 2008.

[48] V. Corby-Harris, A. Drexler, L. Watkins de Jong, Y. Antonova, N. Pakpour, R. Ziegler, F. Ramberg, E. E. Lewis, J. M. Brown, S. Luckhart, and M. A. Riehle. *PLoS Pathog.* 6:e1001003, 2010.

[49] C. S. Lofgren, D. A. Dame, S. G. Breeland, D. E. Weidhaas, G. Jeffery, R. Kaiser, H. R. Ford, M. D. Boston, and K. F. Baldwin. *Am J Trop Med Hyg.* 23:288, 1974.

[50] W. J. Tabachnick, and J. R. Powell. *Nature.* 272:535, 1978.

[51] D. L. Bailey, R. E. Lowe, D. A. Dame, and J. A. Seawright. *Am J Trop Med Hyg.* 29:141, 1980.

[52] H. Townson. *Malar J.* 8 Suppl 2:S10, 2009.

[53] N. Hill, A. Lenglet, A. M. Arnez, and I. Carneiro. *BMJ.* 335:1023, 2007.

[54] B. McElroy, V. Wiseman, F. Matovu, and W. Mwengee. *Malar J.* 8:95, 2009.

[55] C. F. Curtis. *Review of Medical and Veterinary Entomology* 80:543, 1992.

[56] S. P. Frances, D. G. E. Waterson, N. W. Beebe, and R. D. Cooper. *Journal of the American Mosquito Control Association.* 21:480, 2005.

[57] S. J. Moore, A. Lenglet, and N. Hill. *J Am Mosq Control Assoc.* 18:107, 2002.

[58] G. C. Muller, A. Junnila, J. Butler, V. D. Kravchenko, E. E. Revay, R. W. Weiss, and Y. Schlein. *J Vector Ecol.* 34:2, 2009.

[59] B. S. Park, W. S. Choi, J. H. Kim, K. H. Kim, and S. E. Lee. *J Am Mosq Control Assoc.* 21:80, 2005.

[60] J. Zhu, X. Zeng, Yanma, T. Liu, K. Qian, Y. Han, S. Xue, B. Tucker, G. Schultz, J. Coats, W. Rowley, and A. Zhang. *J Am Mosq Control Assoc.* 22:515, 2006.

[61] M. S. Fradin, and J. F. Day. *N Engl J Med.* 347:13, 2002.

[62] S. P. Carroll, and J. Loye. *J Am Mosq Control Assoc.* 22:507, 2006.

[63] S. J. Moore, S. T. Darling, M. Sihuincha, N. Padilla, and G. J. Devine. *Malar J.* 6:101, 2007.

[64] S. J. Moore, N. Hill, C. Ruiz, and M. M. Cameron. *J Med Entomol.* 44:624, 2007.

[65] D. R. Barnard, U. R. Bernier, K. H. Posey, and R. D. Xue. *J Med Entomol.* 39:895, 2002.

[66] A. Roy, M. A. Ansari, and V. P. Sharma. *J Am Mosq Control Assoc.* 7:11, 1991.

[67] D. A. Collins, J. N. Brady, and C. F. Curtis. *Phytotherapy Research.* 7:17, 1993.

[68] C. E. Schreck, and B. A. Leonhardt. *J Am Mosq Control Assoc.* 7:433, 1991.

[69] D. R. Barnard, and R. D. Xue. *J Med Entomol.* 41:726, 2004.

[70] S. S. Barasa, I. O. Ndiege, W. Lwande, and A. Hassanali. *J Med Entomol.* 39:736, 2002.

[71] W. G. Reifenrath, J. J. Olson, U. Vedula, and T. G. Osimitz. *J. Toxicol. Environ. Health. A.*, 72:796, 2009.

[72] Environmental Protection Agency. *P-menthane-3,8-diol (011550) biopesticide registration eligibility document*: https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/related_PC-011550_1-May-00.pdf.

[73] L. S. Nerio, J. Olivero-Verbel, and E. Stashenko. *Bioresource Technology.* 101:372, 2010.

[74] B. C. Clark, T. S. Chamblee, and G. A. Iacobucci. *The Journal of Organic Chemistry.* 49:4557, 1984.

[75] Y. Yuasa, H. Tsuruta, and Y. Yuasa. *Organic Process Research & Development.* 4:159, 2000.

[76] H. E. Zimmerman, and J. English. *Journal of the American Chemical Society.* 75:2367, 1953.

[77] H. Cheng, X. Meng, R. Liu, Y. Hao, Y. Yu, S. Cai, and F. Zhao. *Green Chemistry.* 11:1227, 2009.

[78] W. van der Goes van Naters, and J. R. Carlson. *Nature.* 444:302, 2006.

[79] World Health Organization. *Fact sheets malaria*: http://www.who.int/malaria/.

[80] M. Debboun, S. Frances, and D. Strickman. *History of insect repellents. Insect repellents–principles, methods, and uses*, 1st Ed ed. CRC press: Fort Lauderdale, 2006.

[81] C. Costantini, A. Badolo, and E. Ilboudo-Sanogo. *Trans R Soc Trop Med Hyg.* 98:644, 2004.

[82] J. K. Trigg. *J Am Mosq Control Assoc.* 12:243, 1996.

[83] M. B. Abou-Donia, K. R. Wilmarth, K. F. Jensen, F. W. Oehme, and T. L. Kurt. *J Toxicol Environ Health.* 48:35, 1996.

[84] J. C. Veltri, T. G. Osimitz, D. C. Bradford, and B. C. Page. *J Toxicol Clin Toxicol.* 32:1, 1994.

[85] P. J. Robbins, and M. G. Cherniack. *J Toxicol Environ Health.* 18:503, 1986.

[86] H. Qiu, H. W. Jun, and J. W. McCall. *J Am Mosq Control Assoc.* 14:12, 1998.

[87] A. Tawatsin, S. D. Wratten, R. R. Scott, U. Thavara, and Y. Techadamrongsin. *J Vector Ecol.* 26:76, 2001.

[88] Y. Trongtokit, Y. Rongsriyam, N. Komalamisra, and C. Apiwathnasorn. *Phytother Res.* 19:303, 2005.

[89] Centers for Disease Control and Prevention. *West nile virus*: http://www.cdc.gov/ncidod/dvbid/westnile/RepellentUpdates.htm.

[90] Environmental Protection Agency. *Pesticides*: http://www.epa.gov/pesticides/health/mosquitoes/ai_insectrp.htm.

[91] Y. G. Gillij, R. M. Gleiser, and J. A. Zygadlo. *Bioresour Technol.* 99:2507, 2008.

[92] N. C. Spero, Y. I. Gonzalez, M. A. Scialdone, and D. L. Hallahan. *J Med Entomol.* 45:1080, 2008.

[93] J. Drapeau, M. Verdier, D. Touraud, U. Krockel, M. Geier, A. Rose, and W. Kunz. *Chem Biodivers.* 6:934, 2009.

[94] P. D. Douglas. UK patent GB 2282534 A. 1995.

[95] A. Sikander, M. Ikram ul-Haq, A. Qadeer, and J. Iqbal. *Electron. J. Biotechnol.*, 5:258, 2002.

[96] Government of Canada. *Essential oil-based personal insect repellents - report of an external advisory panel*: https://www.canada.ca/en/health-canada/services/consumer-product-safety/pesticides-pest-management/public/advisory-bodies/essential-oil-based-personal-insect-repellent-external-advisory-panel/report.html.

[97] M. Rowland, T. Freeman, G. Downey, A. Hadi, and M. Saeed. *Trop Med Int Health.* 9:343, 2004.

[98] D. R. Barnard, *Who/cds/whopes/gcdpp/2000.5, global collaboration for development of pesticides for public health, who, geneva*.

[99] D. L. Sudakin, and W. R. Trevathan. *J Toxicol Clin Toxicol.* 41:831, 2003.

[100] A. Badolo, E. Ilboudo-Sanogo, A. P. Ouedraogo, and C. Costantini. *Trop Med Int Health.* 9:330, 2004.

[101] T. J. Naucke, R. Kropke, G. Benner, J. Schulz, K. P. Wittern, A. Rose, U. Krockel, and H. W. Grunewald. *Parasitol Res.* 101:169, 2007.

[102] L. H. Prince. *Microemulsions: Theory and practice*. Academic Press: NewYork, 1977.

[103] G. D. Smith, C. E. Donelan, and R. E. Barden. *Journal of Colloid and Interface Science.* 60:488, 1977.

[104] G. R. Burnett, R. Atkin, S. Hicks, and J. Eastoe. *Langmuir.* 20:5673, 2004.

[105] M. Clausse, L. Nicolas-Morgantini, A. Zradba, and D. Touraud. *Microemulsion systems*. 15. H. L. Rosano, M. Clausse, Marcel Dekker Inc.: New York, 1987.

[106] K. M. Knight, and D. J. Caruana. *Colloids and Surfaces A: Physicochemical and Engineering Aspects.* 301:352, 2007.

[107] P. Bauduin, A. Renoncourt, A. Kopf, D. Touraud, and W. Kunz. *Langmuir.* 21:6769, 2005.

[108] P. Liu, and T. K. Bergstrom. *Journal of Pharmaceutical Sciences.* 85:320, 1996.

[109] A. C. Williams, and B. W. Barry. *Adv Drug Deliv Rev.* 56:603, 2004.

[110] H. Trommer, and R. H. Neubert. *Skin Pharmacol Physiol.* 19:106, 2006.

[111] J. M. Behan, J. N. Ness, P. C. Traas, J. S. Vitsas, and B. J. Willis. U.S. Pat. 5374614.

[112] M. Rieger, L. D. Rhein, and M. Dekker. *Surfactants in cosmetics*. New York, 1997.

[113] A. Kogan, and N. Garti. *Adv Colloid Interface Sci.* 123-126:369, 2006.

[114] P. Combemale, D. Deruaz, D. Villanova, and P. Guillaumont. *Ann Dermatol Venereol.* 119:411, 1992.

[115] P. Granett, and H. L. Haynes. *J Econ Entomol.* 38:671, 1945.

[116] P. Combemale. *Med Trop (Mars).* 61:99, 2001.

[117] B. Ballantyne. *Journal of Applied Toxicology.* 25:248, 2005.

[118] H. I. Leidreiter, and U. Maczkiewitz. *SOFW J.*, 122:674, 1996.

[119] A. E. Voinescu. *Master thesis*, University of Regensburg, 2004.

[120] M. Henneberry, J. A. Snively, G. J. Vasek, and R. Datta. U.S. 6797684. 2004.

[121] B. M. Steib, M. Geier, and J. Boeckh. *Chem Senses.* 26:523, 2001.

[122] U. R. Bernier, D. L. Kline, K. H. Posey, M. M. Booth, R. A. Yost, and D. R. Barnard. *J Med Entomol.* 40:653, 2003.

[123] D. E. Koppel. *The Journal of Chemical Physics.* 57:4814, 1972.

[124] B. J. Frisken. *Appl Opt.* 40:4087, 2001.

[125] World Health Organization. *World malaria report*: http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf?ua=1.

[126] C. Webb, and I. Hess. *Public Health Research & Practice.*

[127] L. I. Goodyer, A. M. Croft, S. P. Frances, N. Hill, S. J. Moore, S. P. Onyango, and M. Debboun. *J Travel Med.* 17:182, 2010.

[128] M. Debboun, and D. Strickman. *Med Vet Entomol.* 27:1, 2013.

[129] L. A. Chaney, R. W. Rockhold, R. W. Wineman, and A. S. Hume. *Toxicol Sci.* 49:306, 1999.

[130] A. Abdel-Rahman, A. M. Dechkovskaia, L. B. Goldstein, S. H. Bullman, W. Khan, E. M. El-Masry, and M. B. Abou-Donia. *J Toxicol Environ Health A.* 67:331, 2004.

[131] M. B. Abou-Donia, L. B. Goldstein, A. Dechovskaia, S. Bullman, K. H. Jones, E. A. Herrick, A. A. Abdel-Rahman, and W. A. Khan. *J Toxicol Environ Health A.* 62:523, 2001.

[132] D. Factsheet. *Journal of pesticide Reform.* 25, 2005.

[133] C. Webb In *Insect repellents handbook, second edition*; CRC Press: 2014, p 213.

[134] S. P. Frances, L. M. Rigby, and W. K. Chow. *J. Am. Mosq. Control. Assoc.* 30:65, 2014.

[135] M. F. Maia, and S. J. Moore. *Malaria Journal.* 10:S11, 2011.

[136] H. Trommer, and R. H. H. Neubert. *Skin Pharmacology and Physiology.* 19:106, 2006.

[137] J. Kalbitz, R. Neubert, and W. Wohlrab. *Pharmazie.* 51:619, 1996.

[138] A. O. Barel, M. Paye, and H. I. Maibach. *Handbook of cosmetic science and technology third edition*. 150. CRC Press: New-York, 2001.

[139] COSMOS-standard *Cosmetics organic and natural standard*: https://cosmosstandard.files.wordpress.com/2014/08/cosmos-standard-v2-21102013.pdf.

[140] I. Manou, L. Bouillard, M. J. Devleeschouwer, and A. O. Barel. *J Appl Microbiol.* 84:368, 1998.

[141] *Ph.Eur.5. Ausgabe, grundwerk, prüfung auf ausreichende konservierung*:

[142] R. A. Rosenthal, S. L. Buck, C. L. Henry, and B. A. Schlech. *Journal of Ocular Pharmacology and Therapeutics.* 22:440, 2006.

[143] B. W. Hugo, and A. D. Russells. *Pharmaceutical microbiology, 7th ed.* Blackwell Science: Oxford, 2005.

[144] B. De Spiegeleer, E. Wattyn, G. Slegers, P. Van der Meeren, K. Vlaminck, and L. Van Vooren. *Pharm Dev Technol.* 11:275, 2006.

[145] A. Cremieux, S. Cupferman, and C. Lens. *Int J Cosmet Sci.* 27:223, 2005.

[146] M. Chorilli, G. R. Leonardi, H. R. Salgado, and M. V. Scarpa. *J AOAC Int.* 94:118, 2011.

[147] P. G. Hubgo, A. O. Onyekweli, and I. Igwe. *Trop. J. Pharm. Res.*, 2:229, 2003.

[148] J. K. Farrington, E. L. Martz, S. J. Wells, C. C. Ennis, J. Holder, J. W. Levchuk, K. E. Avis, P. S. Hoffman, A. D. Hitchins, and J. M. Madden. *Appl Environ Microbiol.* 60:4553, 1994.

[149] H. Masmoudi, Y. L. Dreau, P. Piccerelle, and J. Kister. *Int J Pharm.* 289:117, 2005.

[150] F. Leal-Calderon, and P. Poulin. *Current Opinion in Colloid & Interface Science.* 4:223, 1999.

[151] R. P. Chhabra In *Rheology of complex fluids*; Krishnan, J. M., Deshpande, A. P., andKumar, P. B. S., Eds.; Springer New York: New York, NY, 2010, p 3.

[152] D. R. Batish, H. P. Singh, R. K. Kohli, and S. Kaur. *Forest Ecology and Management.* 256:2166, 2008.

[153] I. S. Al-Adham, E. Khalil, N. D. Al-Hmoud, M. Kierans, and P. J. Collier. *J Appl Microbiol.* 89:32, 2000.

[154] J. Szejtli. *Cyclodextrin technology*. Kluwer Academic: Dordrecht, 1988.

[155] J. Szejtli. *Chem Rev.* 98:1743, 1998.

[156] H.-K. Frömming, and J. Szejtli. *Cylodextrin in pharmacy*. Kluwer Academic: Dordrecht, 1994.

[157] E. M. M. Del Valle. *Process Biochemistry.* 39:1033, 2004.

[158] U. Numanoğlu, T. Şen, N. Tarimci, M. Kartal, O. M. Y. Koo, and H. Önyüksel. *AAPS PharmSciTech.* 8:34, 2007.

[159] Y. Bakkour, G. Vermeersch, M. Morcellet, F. Boschin, B. Martel, and N. Azaroual. *Journal of inclusion phenomena and macrocyclic chemistry.* 54:109, 2006.

[160] I. Bratu, J. M. Gavira-Vallejo, A. Hernanz, M. Bogdan, and G. Bora. *Biopolymers.* 73:451, 2004.

[161] M. Bogdan, M. R. Caira, and S. I. Farcas. *Supramolecular Chemistry.* 14:427, 2002.

[162] A. d. P. Martins, A. A. Craveiro, M. I. L. Machado, F. N. Raffin, T. F. Moura, C. Novák, and Z. Éhen. *Journal of Thermal Analysis and Calorimetry.* 88:363, 2007.

[163] V. Van Axel Castelli, G. Trivieri, I. Zucchelli, L. Brambilla, T. Barbuzzi, C. Castiglioni, M. Paci, G. Zerbi, and M. Zanol. *Journal of Pharmaceutical Sciences.* 97:3897.

[164] P. Xu, L. X. Song, and H. M. Wang. *Thermochimica Acta.* 469:36, 2008.

[165] W. Ciesielski, and J. Kapuśniak. *Journal of Inclusion Phenomena and Macrocyclic Chemistry.* 64:109, 2009.

[166] J. Orgoványi, L. Pöppl, K. H.-Otta, and G. A. Lovas. *Journal of Thermal Analysis and Calorimetry.* 81:261, 2005.

[167] L. Schwingel, D. Fasolo, M. Holzschuh, I. Lula, R. Sinisterra, L. Koester, H. Teixeira, and V. L. Bassani. *Journal of Inclusion Phenomena and Macrocyclic Chemistry.* 62:149, 2008.

[168] P. Job. *Ann. Chim.*, 9:113, 1928.

[169] T. Ackermann. *Berichte der Bunsengesellschaft für physikalische Chemie.* 91:1398, 1987.

[170] H. A. Benesi, and J. H. Hildebrand. *Journal of the American Chemical Society.* 71:2703, 1949.

[171] R. L. Scott. *Rec.Trav. Chem.*, 75:787, 1956.

[172] M. Regiert, and M. Kupka. DE 10200657. 2002.

[173] J. Orgoványi, E. Oláh, K. H-Otta, and É. Fenyvesi. *Journal of Inclusion Phenomena and Macrocyclic Chemistry.* 63:53, 2009.

[174] K. Boonyarattanakalin, H. Viernstein, P. Wolschann, and L. Lawtrakul. *Scientia Pharmaceutica.* 83:387, 2015.

[175] S. Charumanee, S. Okonogi, J. Sirithunyalug, P. Wolschann, and H. Viernstein. *Scientia Pharmaceutica.* 84:694, 2016.

[176] T. Loftsson, and M. E. Brewster. *Journal of Pharmaceutical Sciences.* 101:3019, 2012.

[177] K. E. Jones, N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. *Nature.* 451:990, 2008.

[178] World Health Organization. *Malaria virus*: http://www.who.int/malaria/.

[179] S. I. Hay, C. A. Guerra, A. J. Tatem, A. M. Noor, and R. W. Snow. *The Lancet infectious diseases.* 4:327, 2004.

[180] G. Benelli. *Parasitol Res.* 114:2801, 2015.

[181] W. H. Organization "Implementation of the global malaria control strategy; technical report " World Health Organization: Geneva, 1993.

[182] C. Peterson, and J. Coats. *Pestic. Outlook.*, 54:75, 2001.

[183] E. Lupi, C. Hatz, and P. Schlagenhauf. *Travel Medicine and Infectious Disease.* 11:374.

[184] J. Stinecipher, and J. Shah. *J Toxicol Environ Health.* 52:119, 1997.

[185] *International Journal of Toxicology.* 27:1, 2008.

[186] M. Gloor, B. Senger, M. Langenauer, and J. W. Fluhr. *Skin Res Technol.* 10:144, 2004.

[187] S. Ghosh, D. Kim, P. So, and D. Blankschtein. *J Cosmet Sci.* 59:263, 2008.

[188] S. Ghosh, S. Hornby, G. Grove, C. Zerwick, Y. Appa, and D. Blankschtein. *J Cosmet Sci.* 58:599, 2007.

[189] C. M. de Jongh, M. M. Verberk, S. W. Spiekstra, S. Gibbs, and S. Kezic. *Skin Res Technol.* 13:390, 2007.

[190] J. Hyun-Jun, S. Chan Young, and K. and Kyu-Bong. *Toxicol Res.* 31:105, 2015.

[191] C. Fruijtier-Pölloth. *Toxicology.* 214:1, 2005.

[192] N. Lourith, and M. Kanlayavattanakul. *Int J Cosmet Sci.* 31:255, 2009.

[193] K. Holmberg. *Curr. Opin. Colloid Interface Sci.*, 6:148, 2001.

[194] M. Lukic, I. Pantelic, and S. Savic. *Tenside Surfactants Detergents.* 53:7, 2016.

[195] S. Vaswani "Bio-based succinic acid," SRI Consulting: Menlo Park, California, 2010.

[196] H. Song, and S. Y. Lee. *Enzyme and Microbial Technology.* 39:352, 2006.

[197] Y. Cao, R. Zhang, C. Sun, T. Cheng, Y. Liu, and M. Xian. *BioMed Research International.* 2013:723412, 2013.

[198] S. D. Rodriguez, L. L. Drake, D. P. Price, J. I. Hammond, and I. A. Hansen. *J Insect Sci.* 15:140, 2015.

[199] A. C. Williams, and B. W. Barry. *Advanced Drug Delivery Reviews.* 56:603, 2004.

[200] D. W. Lachenmeier. *Journal of Occupational Medicine and Toxicology (London, England).* 3:26, 2008.

[201] K. P. Wilhelm, G. Freitag, and H. H. Wolff. *J Am Acad Dermatol.* 30:944, 1994.

[202] I. Effendy, and H. I. Maibach. *Contact Dermatitis.* 33:217, 1995.

[203] A. Bodin, T. Fischer, M. Bergh, J. L. Nilsson, and A. T. Karlberg. *Contact Dermatitis.* 43:82, 2000.

[204] V. Dhapte, and P. Mehta. *St. Petersburg Polytechnical University Journal: Physics and Mathematics.* 1:424, 2015.

[205] S. E. Friberg. *Current Opinion in Colloid & Interface Science.* 2:490, 1997.

[206] S. Schreier, S. V. Malheiros, and E. de Paula. *Biochim Biophys Acta.* 1508:210, 2000.

[207] M. A. Rub, A. M. Asiri, N. Azum, and D. Kabir ud. *Journal of Industrial and Engineering Chemistry.* 20:2023, 2014.

[208] S. C. Lee, K. M. Huh, J. Lee, Y. W. Cho, R. E. Galinsky, and K. Park. *Biomacromolecules.* 8:202, 2007.

[209] N. Kapadiya, I. Singhvi, K. Mehta, K. Gauri, and D. Sen. *Int. J. Drug Dev. Res.*, 3:26, 2011.

[210] P. Bauduin, A. Renoncourt, A. Kopf, D. Touraud, and W. Kunz. *Langmuir.* 21:6769, 2005.

[211] A. M. Saleh, and L. K. El-Khordagui. *International Journal of Pharmaceutics.* 24:231, 1985.

[212] J. Y. Kim, S. Kim, M. Papp, K. Park, and R. Pinal. *Journal of Pharmaceutical Sciences.* 99:3953, 2010.

[213] B. K. Roy, and S. P. Moulik. *Current Science.* 85:1148, 2003.

[214] M. P. Bonevaa, K. D. Danova, P. A. Kralchevskya, S. D. Kralchevskaa, S. D. S. D. Ananthapadmanabhanb, and A. Lips. *Colloids and Surfaces A: Physicochem. Eng. Aspects.* 354:172, 2010.

[215] P. A. Kralchevsky, K. D. Danov, C. I. Pishmanova, S. D. Kralchevska, N. C. Christov, K. P. Ananthapadmanabhan, and A. Lips. *Langmuir.* 23:3538, 2007.

[216] D. Balasubramanian, V. Srinivas, V. G. Gaikar, and M. M. Sharma. *The Journal of Physical Chemistry.* 93:3865, 1989.

[217] V. Srinivas, G. A. Rodley, K. Ravikumar, W. T. Robinson, and M. M. Turnbull. *Langmuir.* 13:3235, 1997.

[218] O. R. Pal, V. G. Gaikar, J. V. Joshi, P. S. Goyal, and V. K. Aswal. *Pramana.* 63:357, 2004.

[219] S. Hussan, W. Rowe, and G. J. T. Tiddy. *Handbook of applied surface and colloid chemistry*. 465. J. Wiley: Chichester, 2001.

[220] S. Shimizu, and N. Matubayasi. *J Phys Chem B.* 118:10515, 2014.

[221] World Health Organization. *Mosquito-borne diseases*: http://www.who.int/neglected_diseases/vector_ecology/mosquito-borne-diseases/en/.

[222] World Health Organization. *Fact sheet: World malaria report 2015*: http://www.who.int/malaria/media/world-malaria-report-2015/en/.

[223] S. D. Rodriguez, L. L. Drake, D. P. Price, J. I. Hammond, and I. A. Hansen. *Journal of Insect Science.* 15:140, 2015.

[224] D. R. Barnard "Repellents and toxicants for personal protection. Position paper. Global collaboration for development of pesticides for public health", World Health Organization: Geneva, Switzerland, 2000.

[225] V. Chen-Hussey, R. Behrens, and J. G. Logan. *Parasites & Vectors.* 7:173, 2014.

[226] T. G. Osimitz, and J. V. Murphy. *Journal of Toxicology: Clinical Toxicology.* 35:435, 1997.

[227] K. Van Roey, M. Sokny, L. Denis, N. Van den Broeck, S. Heng, S. Siv, V. Sluydts, T. Sochantha, M. Coosemans, and L. Durnez. *PLoS Neglected Tropical Diseases.* 8:e3326, 2014.

[228] D. K. Badyal, and C. Desai. *Indian Journal of Pharmacology.* 46:257, 2014.

[229] M. C. Scaife. *Int J Cosmet Sci.* 4:179, 1982.

[230] J. Selling, and B. Ekwall. *Xenobiotica.* 15:713, 1985.

[231] T. Okubo, K. Hiraiwa, S. Kinoshita, and M. Watanabe. *AATEX Journal.* 1:2, 1990.

[232] A. Ben Trivedi, N. Kitabatake, and E. Doi. *Agric Biol Chem.* 54:2961, 1990.

[233] K. Chiba, I. Makino, J. Ohuchi, Y. Kasai, H. Kakishima, K. Tsukumo, T. Uchiyama, E. Miyai, J. Akiyama, Y. Okamoto, H. Kojima, H. Okumura, Y. Tsurumi, M. Usami, K. Katoh, S. Sugiura, A. Kurishita, M. Sunouchi, A. Miyajima, M. Hayashi, and Y. Ohno. *Toxicol In Vitro.* 13:189, 1999.

[234] O. P. Flint. *Altern. Test. Lab. Anim.*, 18:11, 1990.

[235] H. Eagle. *J Exp Med.* 102:595, 1955.

[236] M. A. Perkins, R. Osborne, F. R. Rana, A. Ghassemi, and M. K. Robinson. *Toxicol Sci.* 48:218, 1999.

[237] M. K. Robinson, R. Osborne, and M. A. Perkins. *J Pharmacol Toxicol Methods.* 42:1, 1999.

[238] T. Mosmann. *J Immunol Methods.* 65:55, 1983.

[239] R. L. Grant, C. Yao, D. Gabaldon, and D. Acosta. *Toxicology.* 76:153, 1992.

[240] C.-J. Li, and B. M. Trost. *Proceedings of the National Academy of Sciences.* 105:13197, 2008.

[241] P. Anastas, and J. Warner. *Green chemistry: Theory and practice*. Oxford Univ Press: New-York, 2000.

[242] I. T. Horvath, and P. T. Anastas. *Chem Rev.* 107:2169, 2007.

[243] M. Benaglia In *Recoverable and recyclable catalysts*; John Wiley & Sons, Ltd: 2009, p 301.

[244] J. H. Clark. *Pure Appl. Chem.*, 73:103, 2001.

[245] C. J. Li. *Chem Rev.* 105:3095, 2005.

[246] A. Chanda, and V. V. Fokin. *Chemical reviews.* 109:725, 2009.

[247] M.-O. Simon, and C.-J. Li. *Chemical Society Reviews.* 41:1415, 2012.

[248] K. Tanaka, and F. Toda. *Chemical Reviews.* 100:1025, 2000.

[249] F. P. Byrne, S. Jin, G. Paggiola, T. H. M. Petchey, J. H. Clark, T. J. Farmer, A. J. Hunt, C. Robert McElroy, and J. Sherwood. *Sustainable Chemical Processes.* 4:7, 2016.

[250] R. Singh, and S. M. Celin. *J. ecobiotechnol.*, 2:27, 2010.

[251] A. Loupy. *Comptes Rendus Chimie.* 7:103, 2004.

[252] P. Lidström, J. Tierney, B. Wathey, and J. Westman. *Tetrahedron.* 57:9225, 2001.

[253] R. S. Varma. *Green Chemistry.* 1:43, 1999.

[254] R. S. Varma. *Green Chemistry Letters and Reviews.* 1:37, 2007.

[255] G. Kaupp, M. R. Naimi-Jamal, and V. Stepanenko. *Chemistry – A European Journal.* 9:4156, 2003.

[256] A. K. Bose, S. Pednekar, S. N. Ganguly, G. Chakraborty, and M. S. Manhas. *Tetrahedron Letters.* 45:8351, 2004.

[257] R. S. Varma. *ACS Sustainable Chemistry & Engineering.* 4:5866, 2016.

[258] M. Sillanpää, T.-D. Pham, and R. A. Shrestha In *Ultrasound technology in green chemistry*; Springer Netherlands: Dordrecht, 2011, p 1.

[259] R. Konwarh, S. Pramanik, D. Kalita, C. L. Mahanta, and N. Karak. *Ultrasonics Sonochemistry.* 19:292, 2012.

[260] G. Gelbard. *Industrial & Engineering Chemistry Research.* 44:8468, 2005.

[261] S. D. Alexandratos. *Industrial & Engineering Chemistry Research.* 48:388, 2009.

[262] G. Pialoux, B. A. Gauzere, and M. Strobel. *Med Mal Infect.* 36:253, 2006.

[263] G. Pialoux, B. A. Gauzere, S. Jaureguiberry, and M. Strobel. *Lancet Infect Dis.* 7:319, 2007.

[264] M. Vazeille, C. Jeannin, E. Martin, F. Schaffner, and A. B. Failloux. *Acta Trop.* 105:200, 2008.

[265] A. Bianchini, P. Tomi, A. F. Bernardini, I. Morelli, G. Flamini, P. L. Cioni, M. Usaï, and M. Marchetti. *Flavour and Fragrance Journal.* 18:487, 2003.

[266] V. Castola, A. Bighelli, and J. Casanova. *Biochemical Systematics and Ecology.* 28:79, 2000.

[267] P.-G. Rossi, L. Berti, J. Panighi, A. Luciani, J. Maury, A. Muselli, D. d. R. Serra, M. Gonny, and J.-M. Bolla. *Journal of Essential Oil Research.* 19:176, 2007.

[268] U. R. Bernier, K. D. Furman, D. L. Kline, S. A. Allan, and D. R. Barnard. *J Med Entomol.* 42:306, 2005.

[269] M. Geier, and J. Boeckh. *Entomologia Experimentalis et Applicata.* 92:9, 1999.

[270] D. L. Kline, U. R. Bernier, K. H. Posey, and D. R. Barnard. *J Med Entomol.* 40:463, 2003.

[271] R. Deliza, and H. J. H. MacFie. *Journal of Sensory Studies.* 11:103, 1996.

[272] Y. Trongtokit, C. F. Curtis, and Y. Rongsriyam. *Southeast Asian J Trop Med Public Health.* 36:1423, 2005.

[273] J. J. Bray, P. A. Cragg, A. D. C. Macknight, R. G. Mills, and D. W. Taylor. *Lecture notes on human physiology*. Blackwell Scientific Publications: Oxford, 1986.

[274] B. Gibbons. *National Geographic magazine*, 9:324, 1986.

Publications

This thesis included different studies ranging from construction of formulations, microbiological and cytotoxicity studies to new synthesis methods. The thesis was written so that each chapter layout follows the usual convention of a scientific paper: Abstract, Introduction, Experimental Procedures, Results, Discussion and Conclusions. The different studies led to several publications, which are already published or will be submitted and summarized in the following section. Additionally, a patent has been filed and accepted on the synthesis of the new PMD-Succinate repellent active.

Chapter 2
J. Drapeau, M. Rossano, D. Touraud, U. Obermayr, M. Geier, A. Rose, W. Kunz, Green synthesis of *para*-Menthane-3,8-diol from *Eucalyptus citriodora*: Application for repellent products. C R Chim., 2011, 14, 629-635.

Chapter 3
J. Drapeau, M. Verdier, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz, Effective Insect Repellent Formulation in both Surfactantless and Classical Microemulsions with a Long-Lasting Protection for Human Beings. Chem. Biodivers., 2009, 6, 934-47.

Chapter 4
J. Drapeau, C. Paulme, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz, Formulation development and mosquito repellent activity of a skin lotion based on *para*-Menthane-3,8-diol.

Chapter 5
J. Drapeau, C. Paulme, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz, Studies on inclusion complex of *para*-Menthane-3,8-diol with cyclodextrins. Investigations on repellent activity against mosquitoes.

Chapter 6
J. Drapeau, A. Focké, V. Rataj, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz, Synthesis, Characterization of a New Ester Derived From *para*-Menthane-3,8-diol so called PMD-Succinate and Analysis of the Repellent Activity Against Mosquitoes.

Chapter 7
J. Drapeau, A. Focké, V. Rataj, D. Touraud, W. Kunz, Physical and Chemical Properties of the Repellent Active *para*-Menthane-3,8-diol (PMD-S).

Chapter 8
J. Drapeau, D. Touraud, J. Heilmann, W. Kunz, A comparative study of cytotoxic effects of 5 mosquito repellent actives, IR3535, PMD, DEET, KBR3023 and novel PMD-Succinate (PMD-S), on Keratinocyte and HeLa cells.

Chapter 9
J. Drapeau, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz,
Improved Synthesis of the Mosquito Repellent Active PMD-Succinate (PMD-S) with an Eco-Friendly Method.

Chapter 10
J. Drapeau, C. Fröhler, D. Touraud, U. Kröckel, M. Geier, A. Rose, W. Kunz, Repellent studies with Aedes aegypti mosquitoes and human olfactory tests on 19 essential oils from Corsica, France. Flavour Fragr J., 2009, 24, 160–169.

Patent
V. Rataj-Nardello, D. Touraud, W. Kunz, J. Drapeau, A. Rose, Andreas, p-Menthan-3,8-diolderivate und sie enthaltende Insektenschutzmittel, EP 2 439 188.

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When we landed a day of January with Philippe and Chloé in Regensburg, a typical Bavarian city, 20 fresh centimeters of snow, only 2 pages on the Germany travel guide, I would have never imagined to stay for such a long time. And then, this master topic on mosquitoes was more than unusual and intriguing especially when you deeply hate mosquitoes. However, meeting such nice and generous people, discovering the magnificent Bavaria and learning so much, convinced me to stay a little longer than expected to prepare this thesis.

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Herewith I declare that I made this existing work single handed. I have only used the stated utilities.

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Jeremy Drapeau