Emulsions containing essential oils, their components or volatile semiochemicals as promising tools for insect pest and pathogen management

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Emulsions containing essential oils, their components or volatile semiochemicals as promising tools for insect pest and pathogen management

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Abstract

Most of the traditional strategies used for facing the management of insect pest and diseases have started to fail due to different toxicological issues such as the resistance of target organism and the impact on environment and human health. This has made mandatory to seek new effective strategies, which minimize the risks and hazards without compromising the effectiveness of the products. The use of essential oils, their components and semiochemicals (pheromones and allelochemicals) has become a promising safe and eco-sustainable alternative for controlling insect pest and pathogens. However, the practical applications of this type of molecules remain rather limited because their high volatility, poor solubility in water and low chemical stability. Therefore, it is required to design strategies enabling their use without any alteration of their biological and chemical properties. Oil-in-water nano/microemulsions are currently considered as promising tools for taking advantage of the bioactivity of essential oils and their components against insects and other pathogens. Furthermore, these colloidal systems also allows the encapsulation and controlled release of semiochemicals, which enables their use in traps for monitoring, trapping or mating disruption of insects, and in push-pull strategies for their behavioral manipulation. This has been possible because the use of nano/microemulsions allows combining the protection provided by the hydrophobic environment created within the droplets with the enhanced dispersion of the molecules in an aqueous environment, which favors the handling of the bioactive molecules, and limits their degradation, without any detrimental effect over their biological activity. This review analyzes some of the most recent advances on the use of emulsion-like dispersions as a tool for controlling insect pest and pathogens. It is worth noting that even though the current physico-chemical knowledge about these systems is relatively poor, a deeper study of the physico-chemical aspects of nanoemulsions/microemulsions containing essential oils, their components or semiochemicals, may help for developing most effective formulations, enabling the generalization of their use.

Keywords: semiochemicals; nanoemulsions; microemulsions; toxicity; bioactivity; disease management; essential oils

1. Introduction

The impact of diseases transmitted by insects, e.g. dengue, zika, chikungunya, yellow fever or malaria has increased worldwide in recent years [1-4]. On the other side, insect activity also impacts on grain and seeds, which results in constrains to food security and economic loss [5, 6]. Furthermore, human health and environment are affected by different microorganism, such as bacteria or fungi. This has driven important research efforts on seeking new strategies for the management of insect pest, pathogens and diseases. Among such strategies the chemical control is probably the most exploited approach during the last fifty years [7]. Commercially available formulations of pesticides and other biocides are commonly liquid mixtures (aqueous or non-aqueous solutions/dispersions) or solid systems (wettable powders or water-dispersible granules) [8], which contains synthetic molecules.

Paying attention to pesticide formulations, most of the commonly used formulations contain pyrethroids, organophosphates, organochlorines, carbamates, glyphosate, and triazoles. However, the potential risks and hazards for non-target organisms and environment (pollution) associated with the excessive use and misuse of such synthetic insecticides are important barriers to their regulatory approval or when their use is granted, their application conditions undergo periodical revisions [9, 10]. Furthermore, the emergence of cross-resistance against synthetic insecticides in insect pest with medical interest is another important drawback that drives the progressive substitution of conventional insecticides from the pest control programs for eco-sustainable molecules [11-16].

The current trends on integral insect pest and disease management pay special attention to the biopesticide application, i.e. natural products and agents that provoke specific biological response on individuals [17, 18]. However, it should be noted that the meaning of the biopesticide concept remains subjected to a strong controversy. According to the European Commission, biopesticides are pesticides based on microorganisms or natural products, whereas the Environmental Protection Agency (EPA) of USA provides an extended definition of the biopesticide concept, including naturally occurring substances (biochemical pesticides), microorganisms (microbial pesticides) and substances produced by plants containing added genetic material (plant-incorporated protectants, PIPs), which enable the pest control [19]. An extended definition of biopesticides includes any molecule or agent obtained from different organism (plants, bacteria and other microbes, fungi, etc.), with their main advantages in relation to traditional chemical pesticides being: (i) less toxicity; (ii) impact only on the target pest or closely related organisms; (iii) reduced amount results in a strong effectiveness; (iv) fast decomposition, and (v) low exposition, minimizing the pollution risks [19].

A promising alternative for pest control is the use of pesticides based on essential oils (EOs) and their components (EOCs). These molecules are natural products extracted from aromatic plants, having the ability to inhibit the activity of several phytopathogens, human pathogens, and insects [20]. The use of semiochemical compounds, or simply semiochemicals [21] is other alternative with growing interest in recent years for pest control. Semiochemicals (SEs) are chemical compounds enabling the biological communication between living organisms [21, 22]. Insects use semiochemicals to locate mate, host, or food source, avoid competition, escape natural enemies, and overcome natural defense systems of their hosts [23]. Semiochemicals can be divided into two groups depending on whether they result in a biological response on individuals of the same species than the releasing one (pheromones) or on individuals of different species than the releasing one (allelochemicals) [24]. The current interest on the use SEs in pest management are related to their high specificity against insects and their shorter persistence in the environment [25]. On the other hand, the interest for the use of EOs and EOCs is based on these molecules are associated with their role as larvicidal, adulticidal, antifeeding compounds [26-28], their capacity to delay the adult emergence and cause egg mortality [29, 30], their deterrent effects [31, 32], and their arrestant and repellent action [33]. However, the promising properties of EOs, EOCs and SEs cannot hide the many drawbacks associated with their use, including their volatility, limited solubility in water and their thermal and chemical lability (most of these molecules are prompt to oxidation upon exposure to the environment), which make the manipulation and handling of these compounds difficult, and are important drawbacks towards the commercialization of formulations containing this type of molecules [32, 34-37]. Therefore, it is urgent seeking suitable methodologies enabling the design of new eco-sustainable products for integral pest management, which take advantage of the biological activity of EOs, EOCs and SEs.

The current advances of the physico-chemistry of colloidal systems has provided the bases for designing more eco-sustainable formulations for pesticides. These formulations are based on the solubilisation, encapsulation and/or protection of molecules with insecticidal activity on different types of nanocarriers, including soft nanoparticles (e.g. polymers or solid lipid), hard nanomaterials, cyclodextrins, liposomes or emulsions [38-47]. These nanocarriers allow one to concentrate the bioactive compounds within controlled containers, enhancing the stability and availability of the lipophilic molecules (EOs, EOCs or SEs). Thus, it is possible to ensure a more controlled release of the active ingredients, minimizing their losses during the processing and storage [48-53]. Furthermore, the molecular stability of EOs, EOCs or SEs is enhanced due to the retardation of the crystal growth and the minimization of their chemical reactivity (volatility, photodegradation, hydrolysis, oxidation, thermal decomposition or isomerization) [54, 55].

Oil in water (o/w) emulsions containing EOs, EOCs and SEs within the oil phase, alone or in combination with other compounds, have become a very promising tool for overcoming the main drawbacks associated with the handling, storage and application of these molecules [56, 57]. This takes advantage of the ability of emulsions to load and deliver water insoluble molecules. This combined with the high surface area available for interaction and the easiness of their preparation has stimulated the research on the application of emulsions containing EOs, EOCs and SEs as a powerful tool for an integrated pest management strategy [58-60]. Furthermore, the simplicity of the methodologies used for preparation of emulsions containing EOs, EOCs and SEs allows, in many cases, the preparation of the formulations *in situ*, which reduces the cost and pollutant emissions associated with the transport of formulations containing high solvent amounts from stores to the application places, helping on the minimization of the CO_2 footprint. The use of emulsion-like systems as carriers relies many times in the solubilization of the active compound within the disperse phase, commonly the oil one. However, in the particular case of emulsions containing of EOs and EOCs they are commonly introduced directly as the oil phase of the emulsions [61].

This review will be mainly focused on the study of the use of emulsion-like systems containing EOs, EOCs and SEs within the oil phase, and the effectiveness of such emulsions against insect pest. Despite this work is mainly interested in the potential applications against pest, the properties of EOs and EOCs as antimicrobial, antioxidant, anti-inflammatory, analgesic or antiseptic makes necessary to include some discussion about these aspects [62, 63].

2. Formulations for insect pest and pathogen control

Pesticides have been used on the control of animal pests, plant diseases, and weeds since ancient times and are available as dusts, gels, granules, liquids, aerosols, wettable powders, concentrates, aqueous flowable, emulsifiable concentrate (EC), pellets or pre-mixed solutions.

These forms in which pesticides are sold or commercialized are called pesticide formulations. Pesticide formulation also involves the process in which different chemical substances, including the active drug are combined to produce the final product [64]. Therefore, a pesticide formulation contains the active ingredient, which is commonly the molecule enabling the control of the target pest, an organic solvent or mineral clay acting as carrier of the active molecules, surface-active ingredients (thickeners, stickers or spreaders) and/or other ingredients, such as stabilizers, dyes and chemicals to improve or enhance the activity as pesticide of the formulation [65].

The design and development of well sketched vectors, with a well-established and known chemical composition and physical properties, for loading and controlled delivery of active compounds, have focused, in recent years, the research activity of many groups to obtain new efficient platforms that allow overcoming the limitations of the currently used pesticide formulation [66]. During many years, researchers have tried to obtained formulations containing EOs, EOCs and SEs following methodologies that were reminiscent to those used in the preparation of conventional agrochemical formulations for improving the performance of pesticides or enhancing their bioactivity against pests. However, the advances in the formulation technologies occurred in the last decade have provided many tools to the researchers for the optimization of formulations containing of EOs, EOCs or SEs, with different colloidal systems playing a central role in the current trends of the pesticide industry, e.g. surfactant free emulsions [41, 67], nanoparticles [68], nanoemulsions [69, 70], vesicles, liposomes and micelles [71, 72], gels [42, 73], creams[74] and microcapsules [75, 76].

3. Why essential oils?

Essential oils are highly hydrophobic odorous natural compounds with a very limited solubility in water. Essential oils are produced by different organisms for defense, signaling or as part of their secondary metabolism, which makes these compounds a bio-resource for the preparation of eco-sustainable consumers products [77-79]. It is worth mentioning that even though there are different essential oil-like mixtures from animal origin (musks or civet and sperm whale) or produced by microorganisms, the international regulations only consider as essential oils to raw materials from vegetable sources (including flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plants) [80, 81]. In particular, it is widely accepted that essential oils are synthetized by aromatic plants belonging to the angiosperm family, i.e. *Asteraceae, Apiaceae, Lamiaceae, Lauraceae, Myrtaceae, Rutaceae*, and *Verbenaceae* [82]. They can be obtained from different parts of the plants, including roots, stems, leaves or flowers, with their extraction being possible through hydrodistillation, steam distillation, dry distillation, or mechanical pressing procedures [83].The role of EOs in plants can include: (i) attraction of pollinators and other beneficial insects; (ii) thermo-protection, and (iii) defense against pests and/or microorganisms [83]. From a chemical point of view, essential oils are very complex mixtures of different volatile molecules (in some cases more than 60) including terpenes, alcohols, esters, aldehydes, ketones, phenols, ethers, and other minor compounds. However, their biological properties are commonly characterized by two or three components with appear in a relatively high concentration (20-70 wt%). It should be noted that among the compounds comprising essential oils, some of them can present an undesirable role [84].

The use of essential oils (EOs), or their components (EOCs), is widespread in food, perfumery, cosmetics and toiletries, and pharmaceutical industries [37, 79, 85]. This is due to their wide spectrum of biological activities against insect pest, bacteria, yeast, and fungi [86]. In particular, the EOs and EOCs present repellent, insecticidal, and growth-reducing effects on different insects. This activity is mainly the results of their neurotoxic effects to insects through mechanisms addressed to different sites of action: (i) interference with γ-aminobutyric acid receptors (GABA): (ii) modulation of the octopamine synapses, and (iii) inhibition of acetylcholinesterase or P450 cytochrome [50, 83, 87-92]. In addition to the neutrotoxic mode of action, Olmedo et al. [93] reported that the essential oil from *T. filifolia* and (E)-anethole may act as oxidizing agents on insect (lipid peroxidation), and hence their insecticidal activity is not only depend of the acetylcholinesterase inhibition. Essentials oils can also increase the ionic permeability of the cell membrane rather than disturbing cell wall biosynthesis as was reported by Freire et al. [94]. Furthermore, essential oils may affect the integrity of DNA, the mitochondrial respiration chain and the AChE activity in insect as was reported by Castillo-Morales et al. [95] on *Aedes aegypti* larvae.

EOs and their components can be considered a broad family of structurally diverse compounds having different insecticidal and repellent mechanisms, which has fostered their use in formulations of pesticides as active ingredient or co-adjuvant for more than two decades. However, their full potential have not been reached yet because EOs and their components present a high volatility, and a low residuality, i.e. the persistence of their action remains shortly after their application. On the other side, EOs and EOCs are considered safe and eco-sustainable, having a low to moderate toxicity in mammals (the dose needed for resulting in a mortality of the 50% of individuals, LD_{50}, belonging to a rodent populations is in the range 800-3000 $mg \cdot kg^{-1}$) [49, 96, 97].

The promising properties of EOs and their components against different insects cannot hide the many drawbacks reported in relation to their use, among which are included their volatility, poor water solubility and oxidation. These play a very critical role on the biological activity, application and persistence of EOs and EOCs. The use of nanoformulations including EOs or EOCs can solve, at least partially, the above-mentioned limitations, allowing for a reduction of their degradation and increase of their residuality due to the minimization of the evaporation. The inclusion of EOs and EOCs in nanoformulations also provides the bases for their controlled release, making easy their application and handling. Furthermore, an increase on the biological activity of EOs and EOCs can be expected due to the small size of the particles including them, their high surface area and facilitated distribution [96]. This leads to a situation in which many nanoformulations are effective even at very low concentrations. However, the exact mode of action of these nanoformulations is far from clear yet, even though it is generally assumed that their effectiveness is related to their ability to penetrate through the insect cuticle [98, 99]. It is worth mentioning that the promising bioactivity of nanoformulations containing EOs and EOCs cannot hide the uncertainties associated with the possible persistence of the nanoformulations in the ecosystems and their possible toxicity on non-target organism [97]. Thus, the regulatory approval of the use of nanoformulations containing essential oils remain the most important challenge towards the generalization of their use, and consequently towards their commercialization [48, 50, 100].

4. Oil in water emulsions containing essential oils and their components

The use of oil in water (o/w) emulsions, mainly microemulsions and nanoemulsions, including EOs and EOCs have been revealed as a promising approach for improving the dispersion in aqueous environment, penetration and spreading (taking advantage of the low interfacial tension) of these natural compounds, providing the bases for the preparation of insecticides and repellents. This is also possible thanks to the hydrophobic core formed by the surfactant molecules which provides protection to the molecules, avoiding the degradation of their properties and extending their bioavailability for long periods of time [69, 101-108]. Furthermore, the small size of the dispersed droplets containing the oil phase allows overcoming important issues related to their use in the preparation of consumer products, such as the destabilization of the dispersion as result of the gravitational forces. However, the current knowledge about the efficacy of emulsions containing EOs and EOCs against pest remains very limited yet [61, 69, 101-103, 107, 109].

The use of essential oil emulsion based delivery systems is an emerging area on pest control. However, there are limited studies on their application under real field conditions, which offers many challenges to governments, in particular to the regulatory agencies, and industries. It should be remarked that the many properties of essential oils have stimulated a strong development of their application for controlling the proliferation of pathogens, especially in food industry. However, many of the studies related to the effectiveness of such formulations have been performed on model systems, and hence the understanding of their biological activity on real complex matrices remains a challenge [110, 111]. The broad interest of systems containing essential oils is clearly evidenced by the works by Balasubramani et al. [112, 113]. They tested the antibacterial, antioxidant and larvicidal activity of nanoemulsions containing essential oils of *Ocimum basilicum* and *Vitex negundo*, and found a very efficient performance of the dispersions in the three applications.

4.1. Microemulsions containing essential oils and their components

Microemulsions are homogeneous and isotropic colloidal dispersions presenting a low viscosity, optical transparency and thermodynamic stability. The thermodynamic balance associated with the formation of microemulsions can be understood considering the free Gibbs energy balance described considering the following expression [114]

$$\Delta G = \gamma \Delta A - T\Delta S, \tag{1}$$

with γ and ΔA being the oil/water interfacial tension and the area variation associated with the droplet formation, respectively, and T and ΔS the absolute temperature and the variation of the system entropy. The spontaneous formation of microemulsions requires a negative value of ΔG, which can be understood considering that even though the ΔA is very high (formation of many small oil droplets), the low value of the interfacial and the big change of the entropy favor the transition from separate phases to the formation of microemulsions.

The use of microemulsions containing EOs or EOCs can be accounted as ideal candidates for the fabrication of release systems. This is because the combination of their thermodynamic stability with the small size of the oil droplets results in several interesting properties for their use as cargo systems for loading and release hydrophobic substances: (i) ability to disperse EO's in an aqueous phase; (ii) prolong the time of action; (iii) prevent the degradation of the encapsulated compounds, and (iv) increase the solubility of various active substances in water. Furthermore, microemulsions are commonly prepared following a simple procedure, which makes their preparation procedure an easily scalable process [105]. Table 1 summarizes some examples of different microemulsions containing essential oils and their potential applications.

A very important aspect, when formulations based in micromulsions are considered, is to define the pseudo-ternary phase diagram (water/oil/surfactant+co-surfactant) of the considered system. Chaisri et al. [115] studied microemulsions of citronella oil in water stabilized by tween 20 and propylene glycol in different weight ratio (3:1, 2:1, 1:1, 1:2 and 1:3), and found that the optimal mixture of surfactant/co-surfactant was the one with a weight ratio 3:1. The use of such mixture allows maximizing the compositional region in which microemulsions are formed. The obtained microemulsions had a transparent yellow color with droplet presenting an average size in the range 20-40 nm, good long-term stability and low viscosity. The application of the microemulsions as acaricidal against *Rhipicephalus microplus* showed that the concentration of essential oil required to result in a 100% of mortality of larvae was 4-fold lower than the required when the free essential oil is used for the same purpose. Furthermore, the mortality produced by microemulsions in adult individuals was also significantly higher than that associated with the free essential oil (almost 2-fold higher), and even than that associated with the use of the synthetic insecticide cypermethrin. The latter can be understood considering the emergence of resistance to the cypermethrin in *Rhipicephalus microplus*, which makes the microemulsions a promising tool for facing the difficulties in the pest management associated with the emergence of resistance.

Xu et al. [116] also studied the acaricidal activity of microemulsions, in particular they studied microemulsions containing neen oil stabilized using a mixture of tween 80 and sodium dodecyl benzene sulfonate (weight ratio 4:1) as surfactant and hexyl alcohol as co-surfactant. The preparation of the microemulsions was performed by homogenization of the compounds under mild conditions (stirring at 800 rpm during 15 minutes at 40 ºC). This procedure results in the formation of very stable dispersions of globular droplets, which remain stable even after their storage at 4 and 54 ºC during 15 days. Furthermore, the obtained emulsions resist to successive freezing-defreezing cycles without any evidence of destabilization. The exposure of *Sarcoptes scabie var. cuniculi* larvae to the microemulsions containing neem oil evidenced their noticeable acaricidal activity (evaluated as the time required to kill the tested larvae). Navayan et al. [117] also used a mixture of surfactants (tween 80 and span 20 in weight ratio 1:1) combined with a co-surfactant (propylene glycol) for the stabilization of microemulsions of *Eucalyptus globulus* essential oil. The obtained microemulsions contain droplets with a mean size in the range 16-66 nm, and present a viscosity in the range 230-330 cps. Zhang et al. [118] deepen on the different aspects that govern the stabilization of microemulsions containing essential oils. They prepared microemulsions of different EOs stabilized by mixtures of sucrose octanoate ester and soy lecithin, and found that together with the composition of the emulsifying mixture, the chemical nature of the essential plays an essential role on the extension of the microemulsion region. Navayan et al. [117] analyzed the insecticidal activity of *Eucalyptus globulus* essential oil and found a high repellency activity against *Culex pipiens* and *Ochlerotatus caspius* mosquitoes (almost 3 times higher than the free essential oil). This is explained considering that the inclusion of the essential oil within the emulsion droplets reduces the evaporation of the active molecules and delays the release of the essential oil. Thus, the repellent material remain longer time in the environment, giving as result a more prolonged exposure of the mosquito to the repellent molecules. It is worth mentioning that even though the protection associated with the use of microemulsions is relatively high, it remains very far from that obtained from conventional synthetic repellent. However, the use of microemulsions for substituting synthetic repellent minimize the dermal irritation upon application of the formulation. Thus, repellent products based on essential oils may be a safe alternative to conventional products.

Laothaweerungsawat et al. [119] deepened on the different factor that influence the formation of microemulsions, including the surfactant and co-surfactant types, and the surfactant to co-surfactant ratio. For this purpose, they prepared pseudo-ternary phase diagram of mixtures containing different proportions of water, essential oil (*Origanum vulgare* essential oil), different type of co-surfactants (butylene glycol, propylene glycol and glycerin) and various types of surfactant belonging to the polysorbate family (tween 60, tween 80 and tween 85). The found that the compositional range corresponding to the formation of microemulsions was significantly different depending on the specific chemical nature of the used surfactant, with the hydrophilic-lipophilic balance (HLB) of the surfactants and their structure being the two most critical parameters for controlling the extension of the microemulsion region. Furthermore, Laothaweerungsawat et al. [119] also explored the role of the co-surfactant nature and found that the highest the hydrophobicity of the co-surfactant the largest the microemulsion region is. This is explained considering that most hydrophobic co-surfactant can be better incorporated within the hydrophobic region of the surfactant film, leading to a significant reduction of their rigidity and favoring the stabilization of the microemulsions. All the obtained microemulsions were found to be optically homogeneous, transparent, non-birefringent isotropic liquid with a yellowish color. Furthermore, the microemulsions showed a good stability after their exposure to 6 cycles of heating-cooling, without any significant change on their physico-chemical characteristics (appearance, particle size, polydispersity, zeta potential, electrical conductivity, refractive index, pH, and viscosity). Biological test of the microemulsions showed that the encapsulation of the *Origanum vulgare* improved the release profile of carvacrol (main component of the essential oil) which enables their use for transdermal delivery. Furthermore, the microemulsions results in an enhanced anti-inflammatory activity in comparison to the free essential oil, and in particular the inhibitory activity of the microemulsions against the secretion of TNF-α was found comparable to that induced by the dexamethasone, and stronger against the secretion of IL-6.

Hamed et al. [120] used the oil titration method for preparing alcohol free microemulsions of clove oil and its main phenolic component (eugenol), without the use of any co-surfactant. This approach for preparing microemulsions relies on the drop by drop addition of the oil into vials containing a fixed amount of an aqueous solution of the surfactant (tween 20, concentration 5 wt%). This approach allows determining the maximum oil amount that can be included within the dispersion to obtain microemulsions (1.1 and 0.9 wt% for clove oil and eugenol, respectively) and results in the formation of monodisperse microemulsions with droplets presenting a mean size slightly below of 10 nm. The high water content of the obtained microemulsions (around 94 wt%) makes them a promising alternative for the preparation of antimicrobial and antioxidant formulations. The antioxidant activity of the microemulsions was evaluated by measuring the ability to scavenge the stable 2,2-diphenyl-1-picryhydrazyl free radical, with microemulsions of both clove oil and eugenol presenting higher antioxidant activity that the free essential oil. Furthermore, this antioxidant activity is increased with the concentration of phenolic compounds in the microemulsions, i.e. microemulsions of eugenol presents a higher antioxidant character than those containing the whole clove oil. Hamed et al. [120] also tested the activity of their microemulsions against different Gram-positive and Gram-negative bacteria, and found that the latter were more resistant to the treatment with the microemulsions. Nevertheless, the increase of the concentration of phenolic compound results in a significant increase of the susceptibility of both type of bacteria. Purwasena et al. [121] also studied microemulsions of clove oil stabilized by tween 20 for inhibition of the proliferation of *Pseudomonas aeruginosa* films. These studies showed that clove oil microemulsions can prevent, at least partially, the proliferation of *P. aeruginosa* biofilms. Similarly, microemulsions containing *Salvia officinalis and Cinnamomum cassia* oils were found to present a good efficacy for avoiding the proliferation of S*taphylococcus aureus* on surfaces [122]. Furthermore, the inoculation of microemulsions containing clove oil alters the extracellular protease activity and swarming motility of *Pseudomonas aeruginosa* [121]. Clove oils microemulsions have been also studied as antifungal against the proliferation of *Penicillium Digitatum in vivo* and in orange fruit. The results showed that the inclusion of the essential oil within the microemulsions results in a stronger inhibitory activity on spore germination and germ tube elongation than when the pure oil is used. This enhancement of the antifungal activity of the essential oil due to its inclusion in a microemulsions allows one to use lower concentrations, which may be important from the safety and organoleptic perspectives in food protection [123].

Cespi et al. [124] prepared microemulsions of *Smyrnium olusatrum,* stabilized by a mixture of tween 80 and ethanol (surfactant and co-surfactant, respectively), under continuous stirring conditions using the oil titration method, and found the emergence of crystallization of the main component of the essential oil within the oil phase, the isofuranodiene. This problem was overcome by the addition of ethyl oleate, which facilitates the dispersion of the EOs within the emulsion, avoiding their crystallization. Thus, it was possible to prepare microemulsions containing droplets with an average hydrodynamic diameter in the 20-40 nm range and a good long-term stability. The same procedure described above was followed by Pavela et al. [125] in the preparation of microemulsions containing only isofuranodiene. They obtained rather monodisperse microemulsions with the droplets presenting an average hydrodynamic diameter in the 20-30 nm. These microemulsions present a high effectiveness against *Culex quinquefasciatus* larvae, improving the performance of both the whole *Smyrnium olusatrum* essential oil and the free isofuranodiene. Furthermore, these microemulsions evidenced a rather limited ecotoxicity against non-target organism. The crystallization of the isofuranodiene was also found by Pavela et al. [105] in their study of microemulsions containing three different EOs (*Pimpinella anisum*, *Trachyspermum ammi* and *Crithmum maritimum*). The microemulsions of EOs in water were prepared simply by dropping the oil phase (essential oil:ethyl oleate volume ratio 3:1) into an aqueous solution of tween 80 under continuous stirring, which results in microemulsions with a final concentration of the oil phase in the range 1.5-2 wt%. This methodology results in the formation of polydisperse microemulsions, with droplets belonging to two different populations, the first one formed by droplets presenting an average apparent hydrodynamic diameter in the 40-80 nm range, and the second one containing bigger droplets with an average apparent hydrodynamic diameter in the 400-1000 nm range. Despite this polydispersity, dynamic light scattering experiments evidence that most EO was included within the smaller droplets, with the amount of EO included within the bigger droplets being residual. Furthermore, the microemulsions remain stable at least during 6 months of storage. The analysis of the bioactivity of the microemulsions against *Culex quinquefasciatus* larvae shows the acute and chronic toxicity of the formulations (almost the 90% of the larvae die upon treatment with a relative low dose, in the range 1.81-6.48 $mL \cdot L^{-1}$), even though they were not found any significant dependence of the bioactivity of the microemulsions on the chemical nature of the oil phase. Furthermore, the microemulsions results in the inhibition of the adult emergence and an almost negligible toxicity in non-target species. This latter allows considering the microemulsions as a relatively safe alternative for pest control.

Table 1. Summary of microemulsions containing essential oils and their application.

Active compound (concentration)	**Surfactant**	**Purpose**	**Target organism**	**Bioassay**	**Reference**
Pimpinella anisum oil *Trachyspermum ammi* oil *Crithmum maritimum* oil (1.5-2 wt%)	Tween 80	Larvicidal	*Culex quinquefasciatus*	Immersion	Pavela et al. [105]
Isofuranodiene (0.375-0.75 wt%)	Mixtures of Tween 80 and ethanol	Larvicidal	*Culex quinquefasciatus*	Inmersion	Pavela et al. [125]
Eucalyptus globulus oil (5, 10 and 15 wt%)	Mixture of tween 80, span 20 and propylene glycol	Repellent	*Culex pipiens* *Ochlerotatus caspius*	Human-bait	Navayan et al. [117]
Citronella oil (0.039-25 wt%)	Mixtures of tween 20 and propylene glycol	Acaricidal	*Rhipicephalus microplus*	Immersion	Chaisri et al. [115]
Neen oil (10 wt%)	Mixture of tween 80, sodium dodecyl benzene sulfonate and hexyl alcohol	Acaricidal	*Sarcoptes scabie var. cuniculi*	Contact	Xu et al. [116]
Clove oil (1 wt%)	Mixtures Tween 80 and ethanol	Antifungal	*Penicillium Digitatum*	Contact	He et al. [123]
Clove oil	Tween 20	Antibacterial	Different *Gram-positive* and *Gram-*	Contact	Hamed et al. [120]

Eugenol (around 1 wt%)			*negative* bacteria		
Clove oil (5 v/v%)	Tween 20	Antibacterial	*Pseudomonas aeruginosa*	Contact	Purwasena et al. [121]
Salvia officinalis oil *Cinnamomum cassia* oil (5 and 2.5 wt%, respectively)	Tween 20	Antibacterial	S*taphylococcus aureus*	Contact	Campana et al. [122]
Origanum vulgare oil (5 wt%)	Mixtures of tween 60, tween 80 or tween 85 with butylene glycol, propylene glycol or glycerin	Anti-inflammatory	n/a	Inoculation	Laothaweerungsawat et al. [119]

4.2. Nanoemulsions containing essential oils and their components

The use of nanoemulsions instead of conventional emulsions for technological applications is associated with the remarkable kinetic stability and physical stability against coalescence and creaming of the former, and the smaller size of nanoemulsion droplets (5-200 nm), with the latter being an important aspect for the functional activity of the nanoemulsions [106, 126-128]. This makes the nanoemulsions an excellent vehicle for hydrophobic bioactive substances, such as EOs and EOCs, as nanodroplets dispersed in an aqueous medium [60]. Nanoemulsions always present a higher free energy than the demixed stated. Thus, considering the thermodynamic instability of nanoemulsions, i.e. the variation of free energy between the phase separate systems and the nanoemulsions is always positive; their formation is only possible by the application of an external energy input.

The preparation of nanoemulsions can be possible following high-energy and low-energy methods. The possible use of the latter provides an additional advantage of nanoemulsions in comparison to conventional emulsions, even though high-energy emulsification methods such as high-pressure homogenization, microfluidization or sonication are accounted as the most frequently used for their preparation. This type of methods commonly relies on the use of mechanical devices enabling the disruption of the disperse phase into very small droplets. On the other side, the low energy methods used for the preparation of microemulsions rely in the spontaneous dispersion of the disperse phase as droplets within the continuous phase upon the direct mixing of the components. Among the most commonly used low energy methods are included the spontaneous emulsification method (SE), emulsion inversion point method (EIP), phase inversion composition method (PIC) and phase inversion temperature method (PIT). Figure 1 summarizes the most common methodologies used for the preparation of nanoemulsions. The size of the droplets can be tuned by the modification of the HLB of the surfactant used for the stabilization of the nanoemulsions and their concentration, and by tuning different environmental factors [129, 130]. Therefore, the combination of the small droplet size and optical transparency (in some case a slight turbidity can appear) with their high physical stability and their high surface area make nanoemulsions a very interesting carriers for enhancing the bioavailability of active molecules [58, 131, 132]. Table 2 summarizes some examples of different nanoemulsions containing essential oils and their potential applications.

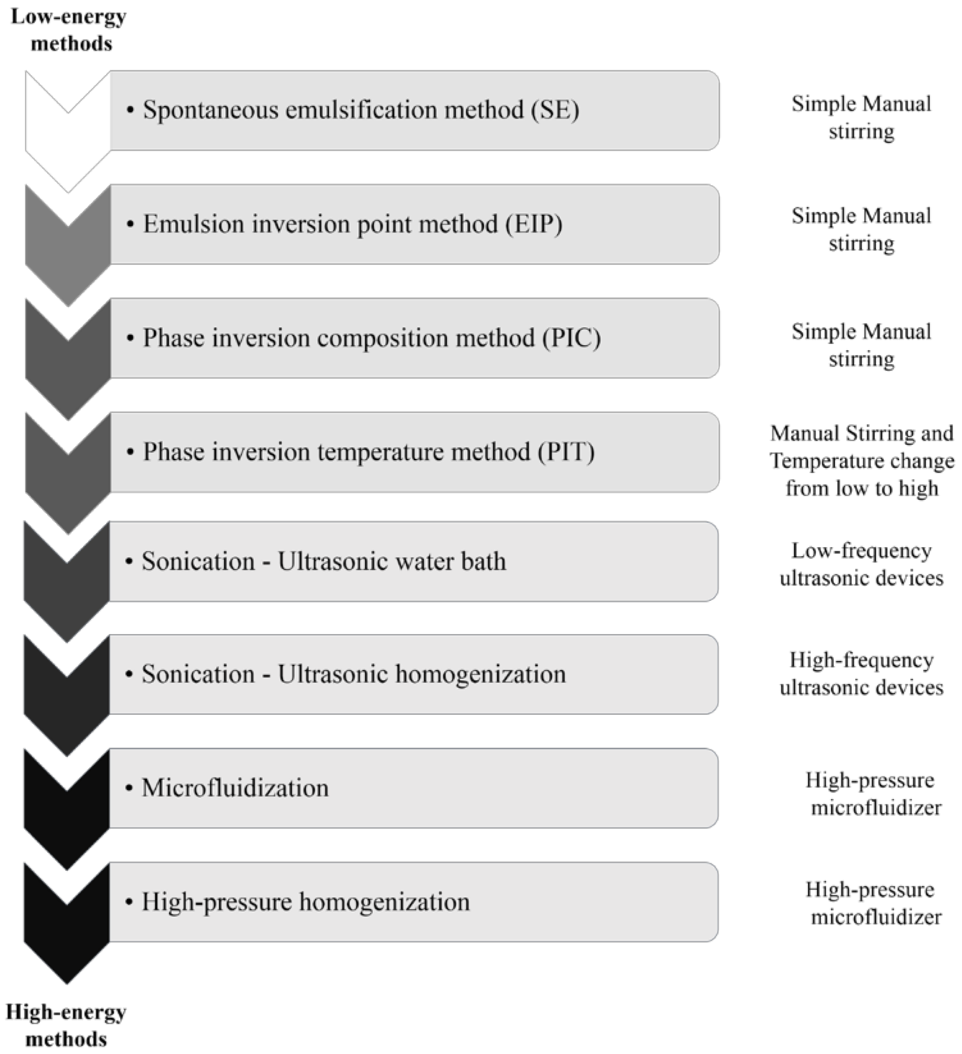

Figure 1.Summary of the methods used for the preparation of nanoemulsions organized depending on the strength of the applied energy.

It should be mentioned that the preparation procedure can be impact decisively on the bioactivity of nanoemulsions as was demonstrated by Salvia-Trujillo et al. [133]. They studied the antimicrobial activity against *Escherichia coli* of nanoemulsions of lemongrass-oil stabilized by alginate, and prepared by two different procedures: ultrasonic homogenization and microfluidization [134]. They found that the intensity of the applied energy input affects to the bioactivity of the nanoemulsions. Thus, nanoemulsions prepared for ultrasonic homogenization evidenced a poorer antimicrobial activity than those prepared using the microfluidization approach. This is explained considering that the high local temperature reached during the ultrasound treatment can be enough to provoke the evaporation of some of the EOCs, which in turn impoverishes the bioactivity of the nanoemulsions. Furthermore, the generation of free radicals as result of the cavitation phenomena occurring during the ultrasonication may contribute to a further degradation of the essential oil. Therefore, even though the application of a high-energy input can be recommendable for enhancing the stability the nanoemulsions, increasing their transparency and reducing the size of the droplets, their use can alter the bioactivity of the encapsulated compounds within the oil phase. Thus, the preparation of bioactive nanoemulsions containing essential oils requires a careful examination of the relationship existing between the physico-chemical properties of the nanoemulsion and their specific bioactivity [135]. The importance of the processing parameter on the preparation of nanoemulsions of essential oil in water was also explored by Liang et al. [136]. They prepared peppermint nanoemulsions stabilized by a succinylated waxy maize starch using a two-step procedure: first, the oil and water phase were premixed using a high-speed homogenizer, and then the coarse emulsions were finely dispersed using a high-pressure homogenizer. The homogeneity and size of the droplets was found to be strongly dependent on the energy input. Thus, the higher the energy input the more homogenous and smaller the droplets are. Pukale et al. [137] also analyzed the impact of the preparation method on the long-term stability of the emulsions and found that the energy input associated with the use of a high energy emulsification procedure (sonication method) favors the droplet break-up in relation to their coalescence, resulting in more stable emulsions with higher long term stability. The above discussion pointed out that the use of high-energy methods favors the stabilization of the emulsions. However, it is also important to control the time of application of the perturbation [138]. This is explained considering that the stabilization of the droplets is the results of two concurrent process: (i) droplet break-up, and (ii) surfactant adsorption onto the droplet-continuous phase interface. Therefore, the stabilization of nanoemulsions requires a time long enough to ensure that a surfactant layer can coat the droplets. However, the time should not be so long to avoid that the droplet break-up can continue, which will increase the area to be coated by the surfactant, and consequently the stability of the emulsions will be compromised. On the other side, an increase of the surfactant concentration does not result in a significant reduction of the size of the droplets for fixed conditions of preparation. Ghosh et al. [130] also reported the importance of the time span of the energy input in the control of the homogeneity and size of the droplets. They found that the longer the energy input the smaller the size of the droplets and their polydispersity is. This is in agreement with the results obtained by Sugumar et al. [135] for nanoemulsions of *eucalyptus* oil stabilized by Tween 80. Jo et al. [60] prepared nanoemulsions of trans-cinnamaldehyde stabilized by tween 20 using high energy homogeneization conditions, which allows them to control the polydispersity and size of the nanoemulsions droplets. Thus, the stronger the energy input the lower the droplet size and their polydispersity are. It is true that high-energy methods are accounted as the most extended for the preparation of essential oil nanoemulsions. However, the use of low energy methods taking advantage of the specific characteristic of the system for obtaining the emulsification are preferably, allowing a significant reduction of the production costs [139].

A very important parameter on the stabilization of nanoemulsions containing EOs or EOCs is the weight ratio between the surfactant and the oil phase as was demonstrated by Wu et al. [140]. They prepared nanoemulsions of thyme oil stabilized with a soluble soybean polysaccharide and found that the increase of the surfactant concentration in relation to that of the oil phase results in a decrease of the droplet size, and a significant hindering of the nanoemulsion creaming. Similar results were found by Anjali et al. [141] for nanoemulsions of neem oil stabilized using tween 20. Furthermore, they found that the larvicidal activity of the nanoemulsions against *Culex quinquefasciatus* can be improved as the size of the droplets was reduced, i.e. with the increase of the surfactant concentration. This agrees with the results obtained by Feng et al. [142] for nanoemulsions containing D-limonene. Furthermore, they found that nanoemulsions were stable to storage at high and low temperatures during long periods, and present a high effectiveness against different fungi (*Pyricularia oryzae*, *Rhizoctonia solani*, *Colletortrichum gloeosporiodes*, and *Phomopsis amygdali*). The importance of the ratio between the surfactant and the essential oil concentrations in the stabilization of nanoemulsions was also found by Sogan et al. [143]. They study of castor oil nanoemulsions stabilized by mixtures of tween 80 and span 20 prepared using ultrasonic emulsification and found that the preparation of stable nanoemulsions was only possible for mixtures in which the surfactant concentration overcome that of the oil phase. Furthermore, they found that the inclusion of the castor oil within the oil phase of o/w nanoemulsions results in a significant enhancement of their bioactivity (almost 30-fold) against the *Anopheles culicifacies* mosquito (vector of malaria disease). Sugumar et al. [144] also highlighted the importance of the weight ratio between the surfactant and the oil phase on the stabilization of nanoemulsions of orange oil in water. They found that even though a minimal concentration of surfactant is required for the nanoemulsion stabilization, an excess of surfactant in the dispersion might be detrimental for the stabilization of the nanoemulsions, resulting in a quick destabilization. Furthermore, Sugumar et al. [144] found that nanoemulsions containing orange oil present anti-yeast activity against *Saccharomyces cerevisiae* both *in vitro* and in a food matrix (apple juice).

Further explorations on the effect of the weight ratio between the surfactant and the oil phase on the stabilization of nanoemulsions were reported by Mossa et al. [145]. They prepared nanoemulsions containing garlic oil by ultrasonic emulsification during 35 minutes and found that the droplet size and the stability of the nanodispersions were correlated to the ratio between the surfactant and the oil phase concentrations. In particular, they found that the formation of stable nanoemulsions was only possible in those mixtures in which the concentration of surfactant was slightly higher than the oil one. Dynamic light scattering showed that nanoemulsions were formed by spherical droplets with sizes in the range 90-100 nm. The high stability of this nanoemulsions was associated with the high value of the zeta potential of the droplets (-30 mV), which provides an electrostatic barrier preventing the coalescence. The obtained nanoemulsions were tested as acaricidal against olive bud mite *Aceria oleae* and olive rust mite *Tegolophus hassani*. Such tests evidenced the improved acaricidal activity of the essential oil due to their inclusion as the oil phase of a nanoemulsion, which was associated with the higher contact area due to the dispersion of the essential oil as nanodroplets. Jo et al. [60] also observed the important role of the increase of the surfactant concentration on the reduction of the size of the oil droplets. Furthermore, they found that the increase of the surfactant concentration results in a transition from opaque nanoemulsions to transparent one, which leads to an increase of the absolute value of the zeta potential of the droplets. Jo et al. [60] also analyzed the release rate from the emulsions and found an increased release rate with the amount of trans-cinnamaldehyde existing within the oil phase of the nanoemulsion. Nanoemulsions containing trans-cinnamaldehyde were found to be very effective for minimizing the proliferation of different bacteria (*Salmonella typhimurium* and *Staphylococcus aureus* except) in both water and watermelon juice, even at a relative low concentration of the essential oil component.

Ghosh et al. [130] found results compatible to the above discussed in relation to the impact of the ratio between the essential oil and surfactant concentrations on the control of the emulsification process. They studied nanoemulsions of eugenol in water stabilized by tween 80, and found that for nanoemulsions containing at fixed amount of eugenol (6 v/v%), a 3-fold increase of the surfactant concentration (from 6 v/v% to 18 v/v%) results in a reduction of the average droplet size from 95 nm to 20 nm. These results are in agreement with the findings by Keyshasalar et al. [146] for nanoemulsions of *Linum usitatissimum* seed essential oil. They found that the higher the concentration of surfactant the smaller the droplet size is. The main role of the surfactant concentration on the droplet size was proved by Chang et al.

[147] for nanoemulsions containing carvacrol. They prepared a series of nanoemulsions with a fixed concentration of oil phase (10 wt%) and increasing amounts of surfactant (tween 80), and found a decrease of the droplet size and of the stability against creaming of the nanoemulsions with the increase of the surfactant concentration. This can be rationalized considering different mechanism: (i) the surfactant concentration affects to the interfacial tension and the mobility of the oil/water boundary where the oil droplets are formed; (ii) a higher surfactant concentration results in a larger number of surfactant molecules diffusing from the organic phase into the aqueous phase when they are put together, with this promoting the formation of finer oil droplets at the oil/water boundary, and (iii) higher surfactant concentrations may favor different structural arrangement of the components of the nanoemulsions, with some of them being advantageous for nanoemulsion production. It is worth mentioning that the long-term stability of the nanoemulsions containing high surfactant concentrations is very poor. Thus, even though the initially formed droplets present a very small size, the nanoemulsions are very unstable and the mean diameter of the droplets undergo a fast growth over few days.

The impact of the specific chemistry of the surfactant on the stabilization of the essential oil component nanoemulsions was also studied by Ghosh et al. [130]. They analyzed the effect of two different surfactants belonging to the polysorbate family (tween 20 and tween 80) on the stabilization of eugenol in water nanoemulsions. They found that the use of tween 80 allows preparing nanoemulsions with smaller droplet size and polydispersity. This difference can be explained in terms of differences on the molecular packing of the non-ionic surfactant at the continuous phase-droplet interface. Chang et al. [147] deepen on the effect of different surfactant belonging to the polysorbate family on the stabilization of nanoemulsions. They found that the HLB of the surfactants plays an essential role on the stabilization of nanoemulsions of carvacrol in water, the higher the hydrophobicity of the surfactant the higher the stability of the nanoemulsions and the smaller their droplets are. However, this does not mean that the stabilization of nanoemulsions with surfactant of the same hydrophobicity results in dispersions containing droplets with the same size. Thus, tween 60 and tween 80, presenting HLB values of 14.9 and 15, respectively, leads to the formation of droplets with mean diameter about 84 and 55 nm, respectively. This difference can be accounted by the different geometries of the surfactant. Thus, even though both surfactant present the same polar head, their hydrophobic tails are different, allowing different packing at the interface and consequently different degree of stabilization. The importance of the interfacial packing of the surfactant molecules was also found a critical parameter on the stabilization of nanoemulsions of *Satureja khuzestanica* essential oil by Mazarei and Rafati [148]. They studied the possible stabilization of nanoemulsions using different combinations of water-soluble surfactants (tween 80 and tween 20) and oil soluble one (span 80 and span 20), and found that mixtures of rigid oil soluble surfactant and flexible water soluble one presents a strong synergetic effect in relation to their ability on the stabilization of nanoemulsions. Furthermore, they found that the size of the droplets obtained upon emulsification and the long-term stability of the nanoemulsions are not correlated. Similar conclusions can be extracted from the studied by Botas et al. [139] where the stabilization of nanoemulsions containing *Baccharis reticularis* and D-limonene with mixtures of tween 80 and span 80 in different weight ratio was explored. Their results evidence the importance of the HLB of the surfactant mixture in the control of the characteristic of the physico-chemical properties of the nanoemulsions and their stability. Furthermore, both nanoemulsions containing *Baccharis reticularis* and D-limonene present a high larvicidal activity against *Aedes aegypti* larvae.

The importance of the chemical nature of the surfactant used for the nanoemulsion stabilization was also reported by Lu et al. [138] in their study of citral emulsions. They prepared nanoemulsions stabilized by different mixtures of Brij 97 and Span 85, and confirm the important role of the HLB value of the surfactant mixture on the control of the stability of the nanoemulsions, existing a characteristic value of HLB enabling the stabilization of emulsions. In particular, the results by Lu et al. showed that surfactant mixtures with HLB below 10 results in opaque nanoemulsions with a high tendency to the phase separation, whereas surfactant mixtures with HLB overcoming the value of 12 allow the stabilization of transparent nanoemulsions with long-term stability. This is important because the reduction of the size of the emulsion droplets was found to favor the retention of the essential oil within the emulsions. The control of the stability using mixtures of surface-active molecules was also evidenced by Xue et al. [149]. They prepared nanoemulsions of thyme oil stabilized by a mixture of sodium caseinate and soy lecithin, and found that the increase of the concentration of the emulsifier mixture allows a better stabilization of the nanoemulsions, with a significant reduction of the droplet size. Furthermore, these nanoemulsions presents a faster reduction of the population of *Listeria monocytogenes*, *Salmonella Enteritidis* and *Escherichia coli* in milk than the free essential oil.

Hu et al. [150] showed that the synergistic effect on the stabilization of nanoemulsions appearing when mixtures of different emulsifier are used may be explained considering the different role of each molecule in the stabilization of the nanoemulsions. They prepared nanoemulsions of eugenol stabilized by mixtures of lecithin and arabic gum and found different behavior on the emulsions stability and droplet size as function of the concentration of each surface active molecule. The increase of the arabic gum concentration (in the range 0-1 wt%) results in a decrease of the droplet size followed by a subsequent increase of the size as the concentration is increased above 1 wt%. This is explained considering that arabic gum can adsorb to the droplet/continuous phase interface. However, the excess of the polymer favors a bridging flocculation and consequently an increase of the droplet size. The role of the lecithin was found to be completely different, the absence of lecithin makes it difficult to stabilize nanoemulsions, i.e. only with arabic gum, and the formation of polydisperse nanoemulsions with large droplets was found. These droplets present a high tendency to sediment. On the other side, the addition of lecithin results in a significant decrease of the droplet size, which is accompanied by an increase of the dispersion turbidity. This is explained considering that the use of mixtures formed by arabic gum and lecithin results in a synergetic effect in relation to the emulsification process, leading the formation of nanoemulsions containing a high concentration of small droplets. The increase of the lecithin beyond a concentration about 0.5 wt% results in a destabilization of the nanoemulsions as result of the excess of lecithin not adsorbed onto the droplets.

Further studies on the effect of the chemical nature of the emulsifier molecules were performed by Li et al. [151]. They studied nanoemulsions of eugenol stabilized by two different surfactants (tween 80 and sodium dodecyl sulfate, SDS) and found that nanoemulsions stabilized by SDS present large droplets (above 1 μm) and undergo a quick phase separation (within the first hour after their preparation). On the other side, nanoemulsions stabilized by Tween 80 do not evidence any significant destabilization, resulting in the formation of droplets with a diameter below 200 nm. These results point out that the stability of the nanoemulsions can be controlled by modulating the interfacial properties as result of the adsorption of surfactants.

Sabbour and Abd El-Aziz [152, 153] explored the impact of nanoemulsions of purslane oil against moths of *Ephestia cautella* and *Sitophilus granaries*, and found a strong sterilizing effect of the nanoemulsions, which remain even after storage of the formulations for more than four months. This latter confirms that the use of nanoemulsions is a good strategy to preserve the integrity of the EOs and EOCs and their biological activity. Nanoemulsions also affect to the viability of the deposited eggs and the adult emergence. Furthermore, formulations of purslane oil as nanoemulsion results in a 3-fold increase of the effectiveness reported for the essential oil, which agrees with the enhanced toxicity of EOs as result of the increase of the contact area occurring when they are dispersed of nanodroplets. Similar effects were found by Nenaah et al. [154, 155] for different *Asteracea* and *Achilea* essential oils against *Callosobruchus maculatus* (cowpea beetle) and *Tribolium castaneum*, respectively. Furthermore, Nenaah et al. [154, 155] found that the lower the surface tension of the nanoemulsions the higher the biological activity of the nanoemulsions is. Adel et al. [156] also explored the effect of nanoemulsions against *Tribolium castaneum* and found that the bioactivity of nanoemulsions containing *Mentha piperita* was more than two times higher than that of the free essential oil, increasing significantly the insect mortality. Furthermore, they found that the treatment of stored grain with the nanoemulsions prevents the emergence of adult insects, without comprising the viability of the stored grain. The impact of nanoemulsions against *Tribolium castaneum* was also verified by Adak et al. [157]. They studied the use of nanoemulsions of eucalyptus oil against the mentioned grain pest and found that the smaller the droplet size the higher the toxicity against the pest is. Similar results were found for the bioactivity of the eucalyptus oil against *Sitophilus oryzae.*

Sabbour and Abd El-Aziz [152, 153] also tested other EOs (castor and mustard oils), and found a lower biological activity against insects, which recall for a correlation between the chemical composition of the essential oil contained within the nanoemulsions and their biological activity. This agrees with the work by Moretti et al. [20]. They studied the impact of very diluted nanoemulsions (water content about 98 wt%) containing different essentials oils, and stabilized with Tween 80 against *Lymantria dispar L.* (Lepidoptera: Lymantridae, gypsy moth) larvae (2nd and 3rd instar) in laboratory bioassays. They found that the mortality of the larvae was strongly dependent on the chemical nature of the essential oils used as oil phase, with emulsions containing *Thymus herba-barona* being accounted as the most effective as larvicidal (80% of larvae die upon 72 hour of treatment with the formulation). This high toxicity is associated with a high content in phenolic compounds, in particular carvacrol [158]. It is worth mentioning that Moretti et al. [20] also found that gelled emulsions favors their biological action due to the enhanced adhesion of the obtained particles to the insect cuticle due to their sticky character which favors a most targeted release of the active molecules.

The importance of the chemical composition of the nanoemulsions oil phase was also verified by Lucia et al. [72]. They explored the impact of nanoemulsions containing different EOCs against *Pediculus humanus capitis*, and found that the toxicity of the nanoemulsions was strongly correlated to the specific chemical nature of the oil phase. Therefore, on the contrary to that what proposed by different authors the size and polydispersity of the droplets was found to play a minor role on their bioactivity (above 50% of the individuals die independently of the essential oil component used as oil phase). This picture was confirmed very recently from the analysis of the larvicidal activity against *Aedes aegypti* of nanoemulsions containing different blends of thymol and carvacrol (two isomeric EOCs) as oil phase [70]. The authors found that the larvicidal activity of the formulations was significantly improved as the thymol content on the oil phase was increased, independently of the size distribution of the emulsions. Furthermore, the biological activity of the combinations of the two essential oils was found to be additive, whereas the replacement of carvacrol for eugenol results in an antagonistic biological interaction [159]. Further studies on the importance of the chemical composition of the oil phase on the biological activity of nanoemulsions were performed by Oliveira et al. [160]. They studied nanoemulsions containing *Lippia sidoides* (main component thymol, 68 wt%) and thymol as oil phase, and found that the chemical composition of the oil phase does not affect significantly to the physico-chemical characteristics of the nanoemulsions. However, the biological activity against *Sitophilus zeamais* was found to be very different depending on the chemical nature of the oil phase. Nanoemulsions containing *Lippia sidoides* results in two-fold higher mortality in insects than those containing only thymol (the dose required to obtain a mortality of the 50% of the individuals, LD_{50}, was found to be 7.10 and 17.08 μg/mg for nanoemulsions with *Lippia sidoides* and thymol, respectively).

The role of the chemical composition of the oil contained in the nanoemulsions was also demonstrated by Ali et al. [161] to play a very important in relation to their toxicity against phytopathogenic fungi (*Rhizoctonia solani* and *Sclerotium rolfsii*). Ali et al. [161] prepared nanoemulsions stabilized by Triton X-100 and Tween 20 by spontaneous emulsification, and performed a carefully characterization of the droplet size and morphology by dynamic light scattering (DLS) and transmission electronic microscopy (TEM), and the stability by centrifugation at 3500 rpm. They reported the formation of physically stable nanoemulsions formed for spherical nanodroplets with diameter in the 8-20 nm range. The activity of the nanoemulsions as phytochemicals was stronger for nanoemulsions containing mixtures of citronella and neem oil as oil phase than for nanoemulsions containing only one of the oils. Pascual-Villalobos et al. [162] explored the preparation of nanoemulsions with up to ten different essential oil and their bioactivity against *Rhopalosiphum padi.* They found that for a constant composition of the nanoemulsion, it is the chemical nature of the essential oil the most critical parameter determining the physico-chemical properties and bioactivity of the dispersions. Thus, nanoemulsions with smaller particles present a significant activity as repellent of *Rhopalosiphum padi*, whereas those with higher particles are almost inactive.

The use of nanoemulsions containing essential oils has been also exploited in the preparation of insect repellent. Nuchuchua et al. [107] prepared nanoemulsions with the oil phase composed by mixtures of three different EOs (citronella, hairy basil, and vetiver oils) and evaluated their repellant activity against *Aedes aegypti* mosquito. They found that the smaller the droplets the stronger the repellency is. This is explained considering the formation of films with higher integrity, which minimizes the evaporation of the EOs and prolongs the repellant activity of the nanoemulsions. Furthermore, the reduction of the droplet size favors the stability of the formulations in agreement with the results by Sakulku et al. [69]. They prepared nanoemulsions with a relatively high citronella content (about 20 wt%) by a high-pressure homogenization process, and found that an increase of the surfactant concentration leads to the decrease of the droplet size and their polydispersity, allowing a more controlled release of the citronella. Furthermore, the addition of glycerol to the aqueous phase is a good alternative for controlling the release kinetics, protection time against mosquito, droplet size, polydispersity index and stability of the nanoemulsions. Thus, the higher the glycerol and surfactant concentration the longer the protection time against *Aedes aegypti* is.

Ribes et al. [163] studied the antifungal activity against *Aspergillus niger* of nanoemulsions containing different EOs, and found that the chemical nature of the stabilizing agent impact (Tween 80 vs anionic whey protein isolate) can impact on the nanoemulsion bioactivity even more than the nature of the EOs. This is explained again considering that the interaction between the emulsion droplets and the target organism controls the action mechanism of the nanoemulsions, which can be understood in terms of different processes: (i) internalization of the oil droplets in the cells due to the passive transport through the cell membrane, which is expected to depend on the mean droplet size; (ii) fusion of the droplets with the cell membrane, which depends on the type of emulsifier and may result in a targeted release; (iii) EO partition between the oil and the aqueous phase, which depends on the chemical nature of the EO and promotes a sustained release over time of the EOs, and (iv) specific interactions between the nanoemulsion droplets and the cell, resulting in an increase of the concentration of EO in the vicinity of cells, depending on the chemical nature of the formulation and emulsifier [164]. Ribes et al. [163] also found that the nanodroplets contained within the nanoemulsions lead to a more important inhibition of the mycelial growth and spore germination than dispersions containing microdroplets. This is explained considering a facilitated penetration through the cell membranes of the target organism. Similar correlations between the size and effectiveness of nanoemulsions were found for the antimicrobial activity of different EOs and EOCs [165, 166]. Moghimi et al. [167] found that nanoemulsions containing *Thymus daenensis* essential oils present a higher effectiveness against *Escherichia coli* than the free essential oil. The mechanism of action was proposed to be associated with the ability of the hydrophobic molecules of the essential oil to disrupt the cell membrane through two different pathways: (i) alteration of the integrity of the phospholipid bilayers, or (ii) interference with the active transport proteins embedded within the lipid membrane. Thus, the changes on the permeability of the membrane lead to the leakage of biomolecules and ions from the cell interior, resulting in a fast cell death. The higher efficient of the nanoemulsions in relation to the free oil is explained considering that the nanometric size of the droplets favored the interaction of the essential oil with the cell membrane. The biocide activity of essential oils nanoemulsions has been also proved against weeds (*Amaranthus retroflexus* and *Chenopodium album*). Harzati et al. [168] showed that o/w nanoemulsions *Satureja hortensis* essential oil (enriched in carvacrol and γ-terpinene) obtained using a low energy method present a strong phytotoxic effect against weed, disrupting the cell membrane and limiting the germination and plant growth. It is worth mentioning that the obtained nanoemulsions present a low polydispersity, and a good stability against destabilization mechanism.

The antimicrobial activity of nanoemulsions containing EOs is probably the most exploited together with their applications in pest control. Topuz et al. [169] found that the use of nanoemulsions containing anise oil (mean droplet diameter about 200 nm) against foodborne pathogens (*Listeria monocytogenes* and *Escherichia coli*) was between 2 and 4 folds more effective than the direct use of the essential oil. Furthermore, it was found that the antimicrobial effect of the essential oil and their stability were extended in the time as result of their protection as nanemulsion droplets. The use of nanoemulsions containing EOs and EOCs have been also tested directly as preservative in food matrices. Bhargava et al. [170] prepared oil-in-water nanoemulsions of oregano oil stabilized by Tween 80 (oil:surfactant ratio 1:2) using a high energy ultrasound approach. These nanoemulsions were found to be very effective on the prevention of the proliferation of different microorganism (*Listeria monocytogenes, Salmonella Typhimurium, and Escherichia coli*) on fresh lettuce.

Further studies on the antimicrobial properties of nanoemulsions against *Escherichia coli* were performed by Salvia-Trujillo et al. [171]. They prepared nanoemulsions with different essential oils (lemongrass, clove, tea tree, thyme, geranium, marjoram, palmarosa, rosewood, sage or mint) and stabilized with tween 80 and sodium alginate. These nanoemulsions were prepared by the application of high shear homogeneization followed by microfluidization to obtain droplets with sizes in the nanometer scale. The stabilization of the droplets by the combination of sodium alginate and tween 80 leads to the formation of negatively charged droplets (zeta potential below -30 mV), which results in an electrostatic stabilization of the nanoemulsions and prevents the coalescence and sedimentation of the oil phase. Furthermore, it was found that the size of the droplets increases with the viscosity of the oil phase, which is ascribed to the stronger disruption forces requires for deforming the droplets during the preparation process. The antimicrobial assays of the nanoemulsions against *Escherichia coli* evidenced again the importance of the chemical composition on the bioactivity of EOs and their components, with nanoemulsions containing lemongrass, clove, thyme or palmarosa essential oils presenting the highest antimicrobial activity. On the other side, the impact of the size of the droplets on the nanoemulsion bioactivity was found to be rather limited. Similar conclusions were obtained for the insecticide and larvicidal activity against different insects of several nanoemulsions [70, 72, 159].

The use of ripening inhibitors is, in many cases, an interesting option to ensure a significant long-term stability of nanoemulsions containing EOs and EOCs, especially when the solubility in water of these molecules may be considered as non-negligible [166]. Thus, it is necessary to seek inhibitors of the Ostwald ripening that do not compromise the bioactivity of the essential oil. A possible alternative is the addition of a water insoluble lipid (soybean, corn or sunflower oil) or a water insoluble surfactant (lecithin) to the nanoemulsions. These ripening inhibitors enhance the stability of the nanoemulsions, even though they can worsen the bioactivity of the nanoemulsions [167]. Chang et al. [166] deepened on the importance of the use of ripening inhibitors. They prepared emulsions of thyme oil in water stabilized by tween 80 and found that such emulsions were highly unstable, presenting evidences of droplet growth and phase separation after 3 days of storage due to the non-negligible solubility of the thyme oil in water. This problem was solved mixing the essential oil with ripening inhibitors (corn oil and medium chain triacylglycerides), which minimizes the destabilization phenomena of the nanoemulsions. For both ripening inhibitors, it was found that the increase of their concentration allows reducing the extension of the droplet growth. This can be explained considering a dual role of the ripening inhibitors: (i) affect the size during the homogenization process, and (ii) affect the droplet stability after homogenization. Furthermore, Chang et al. [166] also found the reduction of the antimicrobial activity of the nanoemulsions as result of the ripening inhibitors, providing a plausible explanation for such behavior. They proposed that antimicrobial agents present a partitioning between oil and aqueous phases, and microorganism cell wall depending on their concentration and oil-water partitioning coefficient. This process is expected to occur very quickly when nanoemulsions are considered due to the small dimensions of the droplets and cells. However, the increase of the amount of oil phase in a nanoemulsion is expected to lead to an increase of the amount of antimicrobial agent included within it, and, hence the amount contained within the aqueous phase containing the microorganism will be reduced, and consequently the interaction between the antimicrobial molecules and the microorganism will be hindered. The medium chain triacylglycerides were also used by Liang et al. [136] as ripening inhibitors in nanoemulsions containing peppermint oil. They ascribed the reduction of the Ostwald ripening of the nanoemulsions to the increase of the viscosity as the medium chain triacylglycerol was added. Similar effects of the addition of medium chain triacylglycerides were reported by Landry et al. [172, 173] for nanoemulsions of carvacrol obtained by a spontaneous emulsification process and stabilized using tween 80. They explored the impact of the composition of the oil phase (carvacrol+ripening inhibitor) on the stability of the nanoemulsions, using for this purpose nanoemulsions with the following composition: 10 wt% oil phase, 10 wt% tween 80 and 80 wt% aqueous phase. The results showed that the composition of the oil phase present a major impact on the formation and stability of the nanoemulsions containing essential oils and their components. The increase of the carvacrol amount in the oil phase, and the consequent decrease of the concentration of the ripening inhibitor (medium chain triacylglycerides), lead to an initial decrease of the mean droplet diameter until a minimum value around 55 nm is reached for nanoemulsions containing a weight ratio carvacrol:ripening inhibitor 1:3. Further increases of the concentration of carvacrol results in an increase of the mean droplet diameter. In particular, nanoemulsions with carvacrol concentration overcoming that of the ripening inhibitor are highly unstable, and undergo a quick fast separation. Figure 2 shows the effect of the oil phase composition, as the weight fraction of carvacrol, on the mean droplet diameter and the aspect of the obtained dispersions as the amount of carvacrol in the oil is phase increased (see inset Figure 2). The results by Chang et al. [147] evidence that the size of the oil droplets is correlated to the composition of the oil phase. This may be associated with the impact of the composition of the oil phase on the properties of the oil phase (viscosity, interfacial tension, and polarity) and of the surfactant (solubility, partitioning, and optimum curvature). An analogous behavior was found by Ryu et al. [174] for nanoemulsions of thyme oil. Furthermore, the chemical nature of the ripening inhibitor also plays a very important role on the stabilization of the nanoemulsions. Thus, the use of medium chain triacylglycerides enables the stabilization of the nanoemulsions, whereas long chain triacylglycerides prevent their formation. Ryu et al. [174] extended the preliminary studies by Chang et al. [147] related to the effect of the chemical nature of the ripening inhibitor on the activity of nanoemulsions. They prepared thyme oil emulsions in water, and used different mixtures of medium chain triacylglycerides and long chain triacylglycerides for the inhibition of the ripening. They found that the increase of the concentration of long chain triacylglycerides in the oil phase results in an increase of the mean diameter of the droplets. However, the increase of the fraction of long chain triacylglycerides in relation to that of medium chain triacylglycerides in the oil phase results in an improvement of the antimicriobial activity of the nanoemulsions.

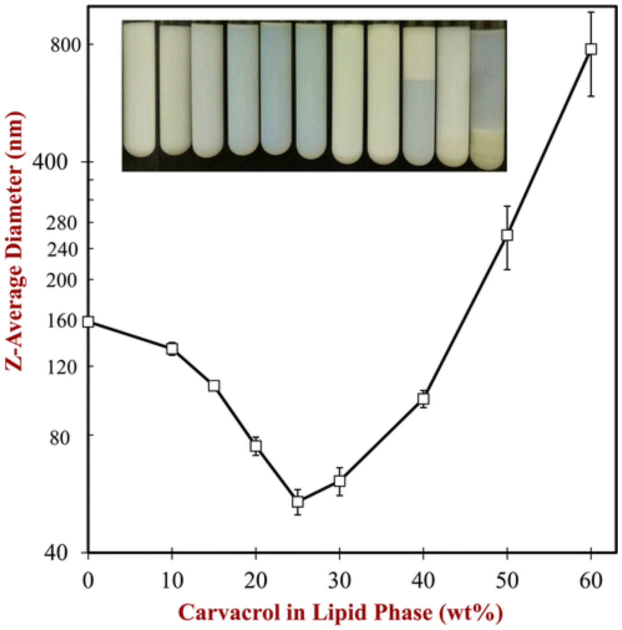

Figure 2. Effect of oil phase composition as the weight fraction of carvacrol in the oil phase on the mean particle diameter for dispersions prepared using 10 wt % oil (carvacrol + ripening inhibitor), 10 wt % tween 80, and 80 wt %. The inset shows a photograph of the aspect of the dispersions containing growing carvacrol concentrations in the oil phase (from left to right of 0, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100 wt %). Reprinted with permission from Ref. [147]. Copyright (2013) American Chemical Society.

The important role of the addition of a ripening inhibitors was especially clear from the studies by Li et al. [151]. They prepared nanoemulsions of eugenol stabilized using SDS which undergo a quick Oswald ripening. This process was retarded by adding increasing amounts of bean oil within the concentration range 0-8 wt%, which leads to a reduction of the size of the droplets. Further increases of the amount of bean oil result in an excessive increase of the viscosity and the interfacial tension that limit the possibility to a further reduction the droplet size. This is the result of a compositional ripening, which is opposed to the Ostwald ripening. It is worth mentioning that the inhibition of the Ostwald ripening associated with the addition of bean oil to eugenol nanoemulsions stabilized by SDS is incomplete, and a growth of the droplets was found after 24 hours of storage. The addition of bean oil to eugenol nanoemulsions stabilized by tween 80 is very different to the behavior found for nanoemulsions stabilized by SDS. Thus, small quantities of bean oil (0-6 wt%) lead to a reduction of the size of the droplets, and the minimization of the Ostwald ripening. Further increases of the concentration of bean oil in the range 6-30 wt% leads to the increase of the droplet size. However, surprisingly the increase of the concentration of bean oil beyond 30 wt% results again in a reduction of the Ostwald ripening. This is explained considering that the compositional ripening can induce a thermodynamically unfavorable difference in droplet composition, which is opposed to the general rule of the Ostwald ripening. This leads to a situation in which the compositional ripening appears as opposed to the Ostwald ripening, resulting in a suppression of the droplet growth. Furthermore, the reduction of the Ostwald ripening resulting from the addition of bean oil to the nanoemulsions stabilized by tween 80 provides a true long-term stabilization to the dispersions. It is worth mentioning that all the nanoemulsions, stabilized either for SDS or for tween 80, present a good antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, with SDS stabilized nanoemulsions being more effective against *Staphylococcus aureus* and those stabilized using tween 80 against *Escherichia coli*. Furthermore, Li et al. [151] found that the increase of the concentration of ripening inhibitors worsens the antimicrobial activity of the nanoemulsions. The above mentioned predominance of the compositional ripening in relation to the Ostwald ripening can be controlled by the choice of the oil phase as was demonstrated by Ferreira et al. [175]. They prepared nanoemulsions of *Lippia alba* essential oil stabilized by Tween 80, and found that the specific composition of the essential oil makes it possible to hinder the Ostwald ripening, resulting in the formation of monodisperse nanoemulsions with long-term stability. The nanoemulsions obtained by Ferreira et al. [175] evidences a strong larvicidal activity against *Culex quinquefasciatus* and *Aedes aegypti* larvae after 48 hours of exposure, with the dose required for obtaining a mortality of the 100% of the larvae being 2-fold lower when the interaction of the nanoemulsiosn with *Aedes aegypti* larvae was considered.

Nanoemulsions combining silicone oil and pine oil stabilized by a mixture of two surfactants (anionic silicone surfactant and tridecyl alcohol ethoxylate) were prepared by Pukale et al. [137] for providing repellency to mosquitoes and antibacterial activity to fabric. The morphology and dimension of the droplets was found to be strongly dependent on both the ratio between the two surfactants in the mixture and that between the silicone and pine oils in the oil phase. The smallest droplets (around 41 nm) were obtained for emulsions with a total surfactant concentration of 8 wt% and 20 wt% oil (silicone oil:pine oil ratio 80:20). In particular, the combination of two surfactants with unique packing and structural properties results in an important synergistic effect on the control of the size of the droplets. The impregnation of fabrics with the nanoemulsions results in structural modification both at the interface and in the bulk. Furthermore, the application of the nanoemulsions in a concentration of 50 g/L results in a repellency to mosquito of the 80% during 30 minutes, whereas the increase of the concentration of the application sample up to 100 g/L takes the repellency up to the 100% (for a period of 30 minutes). Finally yet importantly, the impregnation of the fabric with the nanoemulsion results in a significant reduction of the proliferation of *Staphylococcus aureus* and *Klebsiella pneumoniae* of 21.4% and 54.2%.

Donsi et al. [176] prepared nanoemulsions stabilized by a mixture tween 20-monoolein (1:1 weight ratio) and containing different mixtures of carvacrol and peanut oil (total oil concentration 4 wt%, minimal concentration of peanut oil 1 wt%) as oil phase, using for this purpose the high pressure homogenization technique. The increase of the carvacrol contain results in a destabilization of the nanoemulsions as evidenced the increase of the average hydrodynamic diameter of the droplets, their polydispersity and the turbidity of the dispersions. Thus, it is possible to assume that the peanut oil works as a ripening inhibitor and a minimal amount of this compound is required to minimize the destabilization of the nanoemulsions. However, the optimal essential oil:ripening inhibitor weight ratio was found to be strongly correlated to the specific chemical nature of the essential oil [177]. The obtained nanoemulsions were infused into food matrices for preservation against the proliferation of bacteria, with the infusion process being favored as the size of the droplets is reduced. This is explained considering that the penetration occurs through the membrane pores and intercellular interstices. Furthermore, the chemical composition of the oil phase does not affect significantly to the mass transfer mechanism during the infusion process. The antimicrobial activity was found to be increased with the infusion time due to the higher concentration of biocide molecules incorporated within the matrices. Furthermore, the size of the droplets is the main parameter governing the antimicrobial activity of the nanoemulsios against *Escherichia coli*, the smaller the droplet size the stronger the antimicrobial activity is. The above discussion have pointed out that the presence of ripening inhibitors should be optimized to ensure both the stability of the formulations and their efficacy.

Table 2. Summary of nanoemulsions containing essential oils and their application.

Active compound (concentration)	**Surfactant**	**Purpose**	**Target organism**	**Bioassay**	**Reference**
Purslane oil (0-0.1 wt%)	Triton X-100	Larvicidal/insectide	*Ephestia cautella* *Sitophilus granaries*	Spraying	Sabbour and Abd El-Aziz [152, 153]
Asteracea EOs (Not specified)	Not specified	Larvicidal/insectide	*Callosobruchus maculatus*	Spraying	Nenah et al. [154]
Asteracea EOs (Not specified)	Not specified	Larvicidal/insectide	*Tribolium castaneum*	Spraying	Nenah et al. [155]
Lippia sidoides oil Thymol (20 v/v%)	Procetyl® AWS	Insecticide	*Sitophilus zeamais*	Contact	Oliveira et al. [160]
Eucalyptus globulus oil	Tween 80	Insecticide	*Tribolium castaneum*	Contact	Pant et al. [178]
D-limonene *Baccharis reticularia* oil (2.5 g/L)	Mixtures of tween 80 and span 80	Larvicidal	*Aedes aegypti*	Immersion	Botas et al. [139]
Neem oil (0-20 wt%)	Tween 20	Larvicidal	*Culex quinquefasciatus*	Immersion	Anjali et al. [141]
Castor oil (10 wt%)	Mixtures of tween 80 and span 20	Larvicidal	*Anopheles culicifacies*	Immersion	Sogan et al. [143]
Different EOs (0-1 wt%)	Tween 80	Larvicidal	*Lymantria dispar L.*	Immersion	Moretti et al. [20]
Thymol-carvacrol blends (1.25 wt%)	Poloxamer 407	Larvicidal	*Aedes aegypti*	Immersion	Lucia et al. [70]
Thymol-eugenol blends (1.25 wt%)	Poloxamer 407	Larvicidal	*Aedes aegypti*	Immersion	Lucia et al. [159]
Lippia alba (5 wt%)	Mixtures containing tween 20 or tween 80 and span 20 and 80	Larvicidad	*Culex quinquefasciatus* *Aedes aegypti*	Immersion	Ferreira et al. [175]
Different EOCs (1.25 wt%)	Poloxamer 407	Pediculicide	*Pediculus humanus capitis*	Immersion	Lucia et al. [72]

Mixtures of citronella, hairy basil, and vetiver oils (0-20 wt%)	Alkylpolyglucoside	Repellent	*Aedes aegypti*	Human-bait technique	Nuchuchua et al. [107]
Ten different EOs (5 wt%)	Tween 80	Repellent	*Rhopalosiphum padi*	Spraying	Pascual-Villalobos et al. [162]
Satureja hortensis oil (2 v/v%)	Tween 80	Herbicide	*Amaranthus retroflexus* *Chenopodium album*	Spraying	Harzati et al. [168]
Garlic oil (5 wt%)	Tween 20	Acaricidal	*Aceria oleae* *Tegolophus hassani*	Spraying	Mossa et al. [145]
Citronella-neem oil mixtures (0-10 wt%)	Triton X-100 Tween 20	Antifungal	*Rhizoctonia solani* *Sclerotium rolfsii*	Dropping	Ali et al. [161]
Cinnamon leaf oil Lemon oil Bergamot oil (Not specified)	Tween 80 Anionic whey protein isolate	Antifungal	*Aspergillus niger*	Dropping	Ribes et al. [163]
D-Limonene (0.5 wt%)	Soy lecithin Tween 20 Glycerol monooleate Modified Starch	Antimicrobial	*Lactobacillus delbrueckii* *Saccharomyces cerevisiae*	Dropping	Donsi et al. [165]
Thyme oil (1 wt%)	Tween 80	Antimicrobial	*Escherichia col* *Zygosaccharomyces bailii*	Dropping	Chang et al. [166]
Anise essential oil (Not specified)	Soy lecithin	Antimicrobial	*Listeria monocytogenes* *Escherichia coli*	Dropping	Topuz et al. [169]
Different EOs (1 v/v%)	Mixtures of tween 80 and sodium alginate	Antimicrobial	*Escherichia coli*	Dropping	Salvia-Trujillo et al. [171]

4.3. Surfactantless emulsions containing essential oils and their components

There are some cases in which emulsions can be stabilized even without the introduction of any surfactant, resulting in the so-called surfactant free or surfactanless emulsions (sometimes referred as surfactant free microemulsions). This type of systems results from pseudo-ternary mixtures of oil, water and antisolvent (or amphi-solvent), with the antisolvent being an amphiphilic substance, but not a surfactant, which is at least partially miscible with the aqueous and the oily phases (generally the antisolvent is an alcohol with a short hydrocarbon chain, e.g. ethanol, propanol or butanol) [179]. A general phase diagram for ternary mixtures resulting in the formation of surfactant free emulsions is schematized in Figure 3.

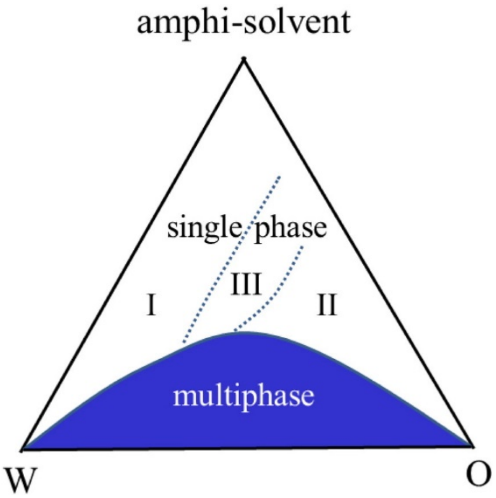

Figure 3. Idealized representation of the pseudo-ternary phase diagram for surfactant-free systems of oil, water, and amphi-solvent. I, II and III represent three different subregions, corresponding to the oil-in-water, water-in-oil, and bicontinuous emulsions, respectively. Reprinted from [179], Copyright (2016), with permission from Elsevier.

The dispersions belonging to the single-phase region are characterized by the formation of optically isotropic and transparent mixtures, whereas the dispersions corresponding to the multiphase region are turbid or present clear evidences of sedimentation or creaming. The single-phase region is commonly characterized by three different subregions, which are associated with different microstructures in the systems. It is worth mentioning that the location and boundaries of the different subregions appearing within the single-phase regions depend on the specific chemical nature of the pseudo-ternary system. Furthermore, the phase diagram can be modified by the ionic strength and pH of the aqueous phase [180-183].

The detailed analysis of the different subregions appearing within the single-phase suggests that true w/o emulsions are formed for dispersions where oil is the main component (subregion II) [180, 181]. On the other side, the characteristic of subregions I and III are very different. The dispersions belonging to the subregion I (enriched in water) may be considered as pseudo-ternary solutions, whereas those belonging to the subregion III, i.e. mixtures with similar concentration of the three components, are formed by small H-bonded aggregates of water-alcohol dispersed in the oil phase (pre-ouzo dispersions) [180-183]. Table 3 summarizes some examples of different surfactantless emulsions containing essential oils and their potential applications.

Lucia et al. [41] proposed that surfactanless emulsions of the water-ethanol-eugenol pseudo-ternary systems may be a good alternative for the fabrication of insect repellent formulations. They make a careful examination of the phase diagram of the pseudo-ternary mixture, which evidenced the formation of a pseudo-single-phase region and a multiphase on, with the latter being characterized by the different partition of the ethanol between the eugenol and water. The transition between the multiphase region and the pseudo-single phase one was found to be marked by the emergence of the pre-ouzo region (for compositions of ethanol in the 30-40 wt% range). Furthermore, the increase of the ethanol concentration favors the stabilization of the dispersions, driving the transition from true surfactantless emulsions to hidroalcoholic solutions. The obtained surfactantless emulsions by Lucia et al. [41, 184] allows the solubilization of a synthetic insecticide (imidacloprid) without any significant modification of the stability of the dispersions. These formulations containing imidacloprid were used in topical and spray assays against *Cimex lectularis* (insecticide resistant colonies) resulting in a mortality of individuals in the 80-85% range. It is worth mentioning that even though both hydroalcoholic solutions and pre-ouzo dispersions can be used for imidacloprid solubilization, the localized structure existing within the droplets of the pre-ouzo dispersions provides a more suitable environment for the dispersion of the insecticide, which improves its availability during the application process [184].

Surfactanless emulsions containing EOCs have been used in the preparation of repellent formulations against insect to replace synthetic repellent [185]. Drapeau et al. [185] studied the repellency of emulsions of the pseudo-ternary system water-isopropanol-para-menthane-3-8-diol against *Aedes aegypti* and found that the repellency upon topical application of the formulations can be extended along 6 hours, which evidence that formulations containing natural actives may present an effectiveness similar to that found in conventional synthetic formulations. In addition, Drapeau et al. [185] also explored the effectiveness of conventional microemulsions and did not found significant different on their activity as repellent in comparison with the surfactantless emulsions.

The above discussion evidences that the applications of surfactantless emulsions in the management of insect pest, pathogens and diseases have been less explored than those involving micro- and nanoemulsions. Nevertheless, the limited environmental impact associated with the absence of surfactant in this type of systems makes them a very promising tool for taking advantage of the bioactivity of essential oils and their components. Thus, the exploration of the bioactivity of surfactanless emulsions containing EOs or EOCs and their correlation to their physico-chemical properties deserve a further exploration to exploit the whole potential of these systems.

Table 3. Summary of surfactantless emulsions containing essential oils and their application.

System	Purpose	Target organism	Bioassay	Reference
Water-ethanol-eugenol (loaded with imidacloprid)	Insecticide	*Cimex lectularis*	Topical Spraying	Cáceres et al. [184]
Water-isopropanol-para-menthane-3,8-diol	Repellent	*Aedes aegypti*	Topical	Drapeau et al. [185]

5. Emulsion-like dispersions containing semiochemicals

Semiochemicals include a wide range of organic molecules, volatile and non-volatile, which are signalling chemicals used to carry information between living organisms, modulating their behaviour. Among volatile semiochemicals are included some essential oil components, therefore EOCs and SEs are closely related chemicals. It is worth mentioning that the same molecule may act as a semiochemical (pheromone, kairomone or allomone) for one insect species, whereas it exhibits bioactivity, as insecticide, repellent, antimicrobial, etc. against another one. Thus, α- and β-pinene contained within the pine essential oil are semiochemicals for siricid woodwasps [186, 187], whereas they present larvicidal activity on *Aedes aegypti* mosquito larvae [188]. Another example of this dual role of certain molecules is found in the methyl eugenol, a natural compound contained in leaves, fruit, stems and flowers, which have an important impact on the insect behavior and the pollination process [189]. This compound can be used as an insect attractant for the control of invasive pests such as the male oriental fruit fly [190], and also exhibit strong insecticidal activities against larvae and adult individuals of mosquitoes [191].

The above discussion was focused on the use of emulsion-like dispersions containing EOs and EOCs in pest control, and in particular the toxicity of such systems against the target pest. However, an integrated pest management strategy should also include non-toxic interventions tools that results in a modification of the behavior of the pests, including the monitoring of popularions, malting disruption and mass trapping of insect species. These tasks can be performed by semiochemicals [21]. The complex biological activity of semiochemicals makes necessary the inclusion of these molecules within platforms, which ensure a controlled release of the volatile biologically active compounds enabling their monitoring during the dispersion in the environment. Furthermore, these volatile and sensitive molecules require to be protected from degradation by UV light and oxygen. Therefore, an efficient incorporation of semiochemicals in integrated pest management strategies requires to fulfill several specifications [192]: (i) the aerial concentration after release must be high enough to be detected by insects; (ii) the release of semiochemicals must be effective during all the period of insect occurrence, and (iii) the production of dispenser must be reproducible.

The semiochemicals can be formulated as sprayable slow-release formulations, where the semiochemical are dissolved in paraffin droplets dispersed in water (paraffin in water emulsions loaded with semiochemicals) [193]. The major advantage of sprayable formulations compared to solid matrix dispensers is that the formulation can be used for the treatment of the entire crop. Atterholt et al. [194] developed emulsions of paraffin in water as controlled release carriers for oriental fruit moth pheromone at concentrations of 2, 4, and 6 wt%. These aqueous emulsion containing the pheromone can be applied using a simple spray devices at ambient temperature, which results in its adhesion on tree bark and foliage, and a progressive release of the pheromone. Furthermore, the pheromone release from paraffin was found to be partition-controlled, providing a constant (zero-order) release rate. The main advantage of this type of formulation is that the controlled release was obtained only by the direct application of the emulsion on the bark o foliage, without any external stimuli. Sprayable formulations were also evaluated by De Vlieger [195] to control Mediterranean Corn Borer (MCB), *Sesamia nonagroides*, through pest mating disruption. They found in outdoor exposure experiments that the release of pheromone was high enough during a time span longer 30 days. These formulation were tested in large scale field experiments by spraying from a helicopter on 5 ha maize fields in Spain, Greece and France. After spraying with the formulations containing the pheromone, it was found that in the first week after spraying the percentage of mated females were lower in the treated field than in the control field, whereas after two weeks less male individuals were captured in traps placed in the treated fields than in those placed traps in the control fields. The effect of mating disruption was also be found when the increase of plant attack was analyzed. In recent years Mafra-Neto et al. [196] designed a new formulation including repellent bark beetle semiochemicals, the so-called SPLAT (Specialized Pheromone and Lure Application Technology). This new formulation consist in an emulsion enabling a controlled-release of semiochemicals [197], which is hand-applied, flowable, and biodegradable. Furthermore, it allows adjusting the size of each release point (dollop) almost at will. The possible application of release points (dollops) at high densities with a desired distribution within the field was found on the origin of the high levels of tree protection against *Dendroctonus ponderosae* [198]. The technology SPLAT verb® is currently used to control *Dendroctonus ponderosae* [199], and the SPLAT ACP Repel® one as a repellent for the Asian citrus psyllid (ACP), *Diaphorina citri* [200]. Several commercial SPLAT® mating disruption products are currently commercialized by ISCA Technologies, Inc (U.S.A) and ISCA Tecnologias Ltda. (Brazil) against different insect pests, including *Grapholita molesta* (SPLAT®OFM), *Cydia pomonella* (SPLAT®Cydia), *Epiphyas postvittana* (SPLAT®LBAM), *Lymantria dispar* (SPLAT®GM), *Tuta absoluta* (SPLAT®Tuta), *Pectinophora gossypiella* (SPLAT®PBW). This type of emulsions has been also evaluated in the field of hematophagous vectors of disease, such as mosquitoes larvae (SPLAT®BAC) and *Ixodes scapularis* (SPLAT®TK) [4].

6. Concluding remarks

This work explores the potential application of nanoformulations containing EOS, EOCs or SEs as an effective tool for management of insect pest and pathogens, with their control being essential for the prevention of diseases. In particular, the use of oil in water nano/microemulsions presents a very central role in the fabrication of efficient formulations because it enables the dispersion of poor-water soluble substances, such as EOS, EOCs or SEs, in an aqueous media, protecting them simultaneously of the detrimental effects associated with their exposure to the environment. Therefore, the inclusion of EOS, EOCs or SEs within the oil phase of nano/microemulsions has become a simple and low cost strategy for taking advantage of the biological activity of these molecules.

The current widespread of the use of nano/microemulsions for taking advantage of the bioactivity of molecules can be considered the result of different aspects: (i) provide a suitable encapsulation platform for molecules with poor physico-chemical stability; (ii) can be used in the design of delivery systems for controlled and sustained release of molecules; (iii) allow a quick interaction of the molecules with the target sites due to the small size of the droplets; (iv) limited creaming preventing destabilization processes (creaming or sedimentation, and (v) their stability and properties depends on both the preparation process and the ingredients.

This review has evidenced that nano/microemulsions containing EOs, EOCs or SEs are very promising tools for pest and pathogen control. However, there are important drawbacks associated with their use. One of the most important drawback for the nano/microemulsion application is related to the use of surfactants for their stabilization. These surfactants are classified as potentially hazardous and toxic substance for non-target organism and environment. This makes the use of nanoemulsions preferable to that of microemulsions as result of the lower concentration of surfactant required for obtaining the former dispersions, which results in a better toxicological profile. Furthermore, the potential impact of size and surface area on the biokinetics behavior and properties of the active compounds is an important limitation to the regulatory approval of formulations based in nano/microemulsions. Despite the above mentioned drawbacks, the efficacy of nano- and microemulsions containing EOS, EOCs or SEs against insect pest and pathogens makes necessary further research efforts for the development of commercial products taking advantage of the current know how. Therefore, it is necessary to close the gap between the laboratory assays and the real field applications, and it is here where the use of a physico-chemical approach based on the current advances of the colloid and interface science can help towards the design of commercial products based on nano/microemulsions containing EOS, EOCs or SEs. This requires a careful examination of the correlations existing between the bioactivity of the nanoformulations and their physico-chemical properties (see Figure 4 for a summary of some important physico-chemical aspects with importance on the preparation of dispersions for insect pest and pathogen control) to exploit the whole potential of these systems for control the proliferation of insect pest and pathogens, reducing the propagation of diseases. This review has tried to present a comprehensive discussion of the most recent advances on the use of nano/microemulsions containing EOS, EOCs or SEs trying to provide a physico-chemical description of the principles underlying their stabilization, which can be used for the development of new nanoformulations of EOS, EOCs or SEs with real field applications.

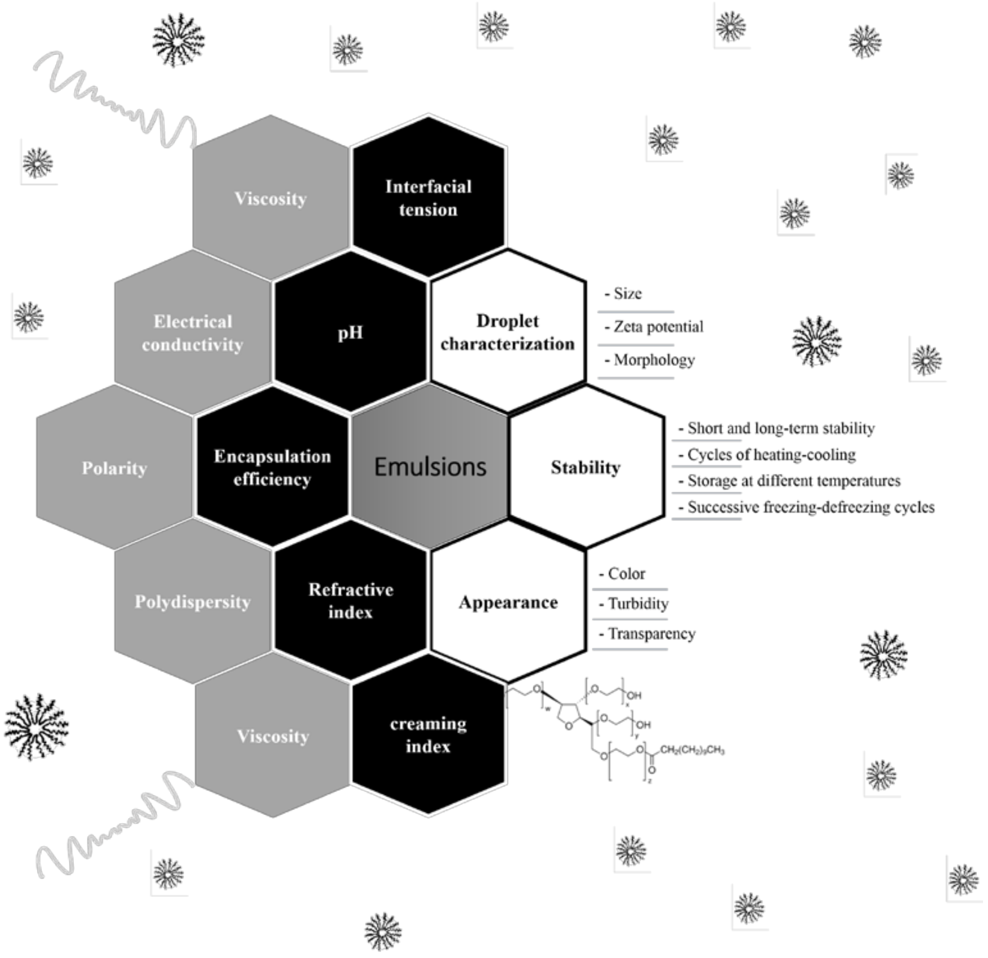

Figure 4. Summary of some physico-chemical aspects with importance in the design of formulations for insect pest and pathogen control.

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