universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Influence of light on essential oil constituents“

verfasst von / submitted by

Marie-Christine Čudlik

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magistra der Pharmazie (Mag.pharm.)

Wien, 2019 / Vienna, 2019

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Univ.-Prof. i.R. Mag. Dr. Gerhard Buchbauer

Acknowledgment

I would like to express my deepest gratitude and appreciation to…

…my supervisor, Univ.-Prof. i.R. Mag. Dr. Gerhard Buchbauer, for his expert guidance and for always having an open door.

…my dad, who always encouraged me in who I am and what I do.

…my mum, who taught me to have dreams and to pursue them.

…my grandparents for their unwavering belief in me.

…my friends and brothers, for all their emotional and moral support.

Abstract

The aim of this diploma thesis was to collect knowledge on the physicochemical conversions that come into action when light interacts with essential oils and their components. Stability of essential oils depends on various external factors such as oxygen availability, temperature and light irradiation, given that ultraviolet and visible light are capable of initiating and accelerating degradation processes. Photochemical reaction pathways include, among others, photoxidation, -polymerization and double-bond isomerization, and can lead to loss of organoleptic properties like odor and flavor, resinification, changes of biological activity and even potentially dangerous effects, such as photoallergic skin responses, phototoxicity and phototumorigenicity. Phototoxicity as an unwanted property of essential oils and their constituents can be assessed by several *in vitro* methods, among them are the validated 3T3 neutral red uptake assay, the photohaemolysis test and reconstituted 3D human skin models.

Abstract

Das Ziel der vorliegenden Diplomarbeit war der Informationsgewinn über die physikalisch-chemischen Umwandlungen, die bei der Interaktion von Licht mit ätherischen Ölen und deren Komponenten stattfinden. Die Stabilität von ätherischen Ölen ist von verschiedenen äußeren Einflüssen wie Sauerstoffverfügbarkeit, Temperatur und Lichteinwirkung abhängig, da ultraviolettes und sichtbares Licht Zerfallsprozesse initiieren und beschleunigen können. Photochemische Reaktionsmechanismen umfassen beispielsweise Photoxidation, -polymerisation und Isomerisierung von Doppelbindungen und können zum Verlust der organoleptischen Eigenschaften wie Geruch und Geschmack, sowie zu Verharzung, Änderungen der biologischen Wirksamkeit und sogar potentiell gefährlichen Wirkungen führen, wie etwa photoallergischen Hautreaktionen, Phototoxizität und Phototumorigenizität. Phototoxizität als unerwünschte Eigenschaft von ätherischen Ölen und deren Bestandteilen kann mithilfe einiger *in vitro* Methoden bestimmt werden, darunter 3T3-Neutralrot-Test, Photohämolyse und rekonstruierte humane 3D Hautmodelle.

Index

1. Introduction

Light is a key foundation for life. It is essentially electromagnetic radiation, and as such, provides energy. Especially the UV range of the electromagnetic spectrum, which is split up into UV-A (315–400nm), UV-B (280–315nm) and UV-C (100–280nm) is rich in energy, however, only UV-A and a fraction of UV-B reach the earth´s surface, as UV-C is completely absorbed by the planet´s ozone layer and atmosphere (WHO, 2018). Ultraviolet radiation is known to have both positive and negative effects, for it is important for the biosynthesis of vitamin D3 in the human body, but can also be a dangerous mutagenic agent. It has sufficient energy to break chemical bonds, creating cleavage sites which can react with oxygen, causing degradation (Pelzl et al., 2018).

Plants need light. They use the energy coming from the sun for chemical reactions, producing primary metabolites, for instance amino acids and carbohydrates, and secondary metabolites, such as essential oils (= EOs) (Heldt/Piechulla, 2011; Prins et al., 2010), which serve them as attractants for insects or as defense compounds against herbivores and pathogens (Heldt/Piechulla, 2011). Various genetic and environmental factors affect the composition and yield of the EO, amongst them the intensity, duration and wavelength of the irradiation to which the plant is exposed (Maffei et al., 1999; Kumari et al., 2009; Ivanitskikh/Tarakanov, 2014). The amount of volatile compounds produced is lowest during the time of the year with the lowest temperature and least hours of sunlight (Figueiredo et al., 2008).

Not only does irradiation affect the living plant, but also the finished product, the EO, once it is situated outside the protective compartments of the plant. When photosensitive EO constituents are submitted to electromagnetic radiation, the molecules may experience photoexcitation, which means they reach an excited state by absorbing the ultraviolet, visible or infrared light (IUPAC, 1997). Usually they are initially transferred to an excited singlet state with a short lifespan, followed by fast radiationless relaxation, which leads via intersystem crossing to an energetically lower excited triplet state (Van den Bergh, 1986). As a consequence of the molecule´s excitation, its oxidative and reductive properties are enhanced, increasing the possibility of electron transfer processes (Ochsner, 1997). Many different photoreactions are possible due to the chemical heterogeneity of EOs. They consist of 20 to 200 single substances (Hänsel/Sticher, 2010), although mostly of two to

three main constituents (20-95%) and others only in trace amounts (Shabaan et al., 2012). The main chemical components of most EOs are terpenoids (like monoterpenes and sesquiterpenes) as well as phenolic compounds, all of them characterized by low molecular weight (Dhifi et al., 2016). In case of oxygen availability, photoxidation is the most important possible photochemical reaction. Furthermore, double-bond isomerization, photopolymerization, Diels-Alder photocycloaddition and photoepoxidation are prevalent transformation pathways for EO constituents under influence of light. Often, neither light nor oxygen alone can trigger chemical reactions in EOs; they work synergistically (Li et al., 2016). Some transformations occur only when energy is provided in form of radiation and oxygen can be taken from the air surrounding. This close relationship between these two factors renders it difficult to differentiate between them and to draw a line as to which is more important in triggering reactions.

The question is, which consequences the various photoalterations have on the EOs and their constituents. Due to their wide-ranging effects, they are ubiquitous in their application as odorants, flavoring substances and antioxidants in the food and liquor industries (Castro et al., 2010), and furthermore as expectorants, stomachics, aroma correctors and many more in the pharmaceutical sector. EOs have been found to be antioxidant, anti-inflammatory (Miguel, 2010; Buchbauer/Erkic, 2016), insect repellent (Chellappandian, 2018), antimicrobial and antiviral (Bakkali et al., 2008), spasmolytic, carminative, sedative (Lis-Balchin/Hart, 1999), hepatoprotective, anticarcinogenic (Morita et al., 2003; Raut/Karuppayil, 2014); they even exhibit wound-healing (Pérez-Recalde et al., 2018), antidiabetic and lipid lowering effects (Habtemariam, 2018). Due to their range of effects and their tolerability they are widely used and therefore an eventual impact on their safety and/or organoleptic properties is of great interest.

Abundant research has been conducted on the manifold useful properties of EOs, but literature on their stability under different storage conditions is scarce, especially on the topic of light-induced transformations.

Therefore, the aim of this treatise was to summarize the physicochemical interactions of light and EO constituents that may occur during production, storage and use of EO. Also, a thorough research was conducted in the field of phototoxicity of EO components, because many EOs have dermal applications and it is therefore important to look into their potential

harmfulness when they could undergo photochemical changes when in contact with direct or indirect sunlight.

2. Mechanisms of photodegradation

According to the IUPAC gold book of definitions (1997), photodegradation is "*the photochemical transformation of a molecule into lower molecular weight fragments, usually in an oxidation process*." It is one of the operations that can change the chemical composition of EOs under storage, transportation or use, causing alterations in organoleptic properties, medicative potency like antimicrobial activity, and toxicity patterns (Beltrame et al., 2013).

Most chemical reactions occur with the participating molecules residing in the ground state, a stable electronic state. Photochemical reactions, on the other hand, are characterized by a shift to excited singlet states due to absorption of the energy of a light quantum, followed by intersystem crossing to triplet states, which possess modified electron spins, enabling different reaction mechanisms (Kayyat/Roselin, 2018).

Photochemical degradation is, considered quantitatively, one of the most important reactions in nature. When trace substances in the troposphere interact with sunlight, reactive species, mostly radicals, are formed. These can oxidize hydrocarbons and their derivatives, resulting in indirect photochemical degradation, the products of which are ultimately carbon dioxide and water, thus leading to the natural purification of the atmosphere. Direct photochemical degradation, on the other hand, is the absorption of a light quantum followed by immediate oxidation or rearrangement, and is relatively rare, due to the high activation energy (Simmler, 2012).

Photodegradation can also take effect for EOs and their components. After being extracted from the plant, the substances can undergo the reaction while being processed, transported, stored or used, thus being afflicted in their organoleptic properties and possibly as well in their safety and effects on the user. Depending on the exact circumstances of product handling, different mechanisms of photodegradation can come into action. For instance, when oxygen is available, photoxidation is most likely to occur, whereas in the case of hydrogen peroxide presence, epoxidation is possible. These mechanisms are being discussed in this chapter.

2.1. Double-bond isomerization

Back in 1983, Toda et al. investigated the photolysis of both **jasmine absolute oil** and one of its main constituents, benzyl benzoate, in ethanolic solution. They used a high-pressure mercury lamp (HPML) and a low-pressure mercury lamp (LPML), respectively, for irradiation of the samples, and delivered a proposed degradation pathway and detailed data about the changes in composition over time. The compounds containing a chain double-bond experienced a *Z-E* isomerization reaction, resulting in a fairly large amount of *trans*-configured products. This isomeric shift occurred under both HPML and LPML irradiation, but to a greater extent under the latter. The affected compounds were 3-methyl-2-(*cis*-2-pentenyl)-2-cyclopenten-l-one (= *cis*-jasmone, compound 1 in Fig.1), *cis*-3-hexenyl benzoate, *cis*-7-decen-5-olide, phytyl acetate, geranyl linalool, and phytol (Toda et al., 1983). Tateba et al. (1993) subjected a solution containing *cis*-jasmone to irradiation with a high-pressure mercury lamp for 20 hours in methanol under nitrogen atmosphere, and came to the same result regarding the isomerization reaction, but also detected two di-π-methane rearrangement products (compounds 3a/b in Fig.1) as well as two intramolecular

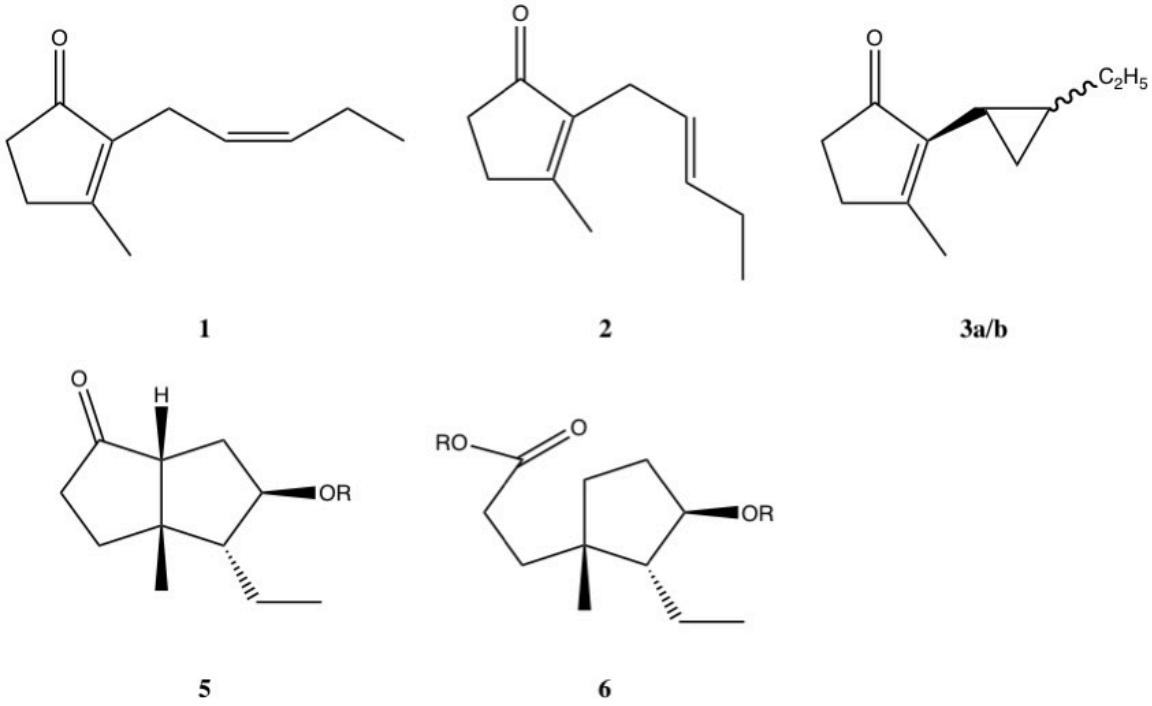

Figure 1: Photoreaction products of cis-jasmone, R= CH_3, C_2H_5 (depending on solvent) (adapted and newly drawn from Tateba et al., 1993)

cyclo-adducts (compounds 4 and 5 in Fig.1). When ethyl acetate was used as solvent, only compounds 2, 3a and b were generated, however, the yield of products 3a and b (83%) increased considerably compared to ethanol (68%) or methanol (69%) (Tateba et al.,1993).

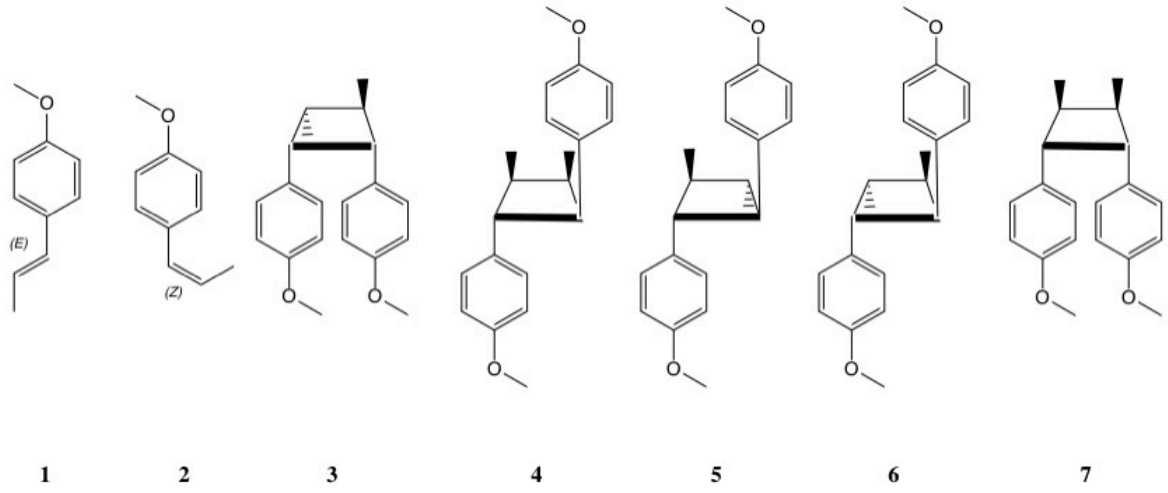

Figure 2: Photoreaction products of trans-anethole (adapted and newly drawn from Castro et al., 2010)

***trans*-Anethole** (structure 1 in Fig.2) is one of the major components of anise, clove, thyme and cinnamon EO and often used in food and liquor industries. Its use is controversial, for upon UV irradiation, *cis*-anethole (structure 2 in Fig.2) is formed, which is toxic. Castro et al. investigated the transformation of *trans*-anethole in toluene subjected to UV radiation and with help of GC-MS found that (1a,2a,3b,4b)-1,2-bis(4-methoxyphenyl)-3,4-dimethylcyclobutane (structure 7 in Fig.2) was the most abundant constituent of the mixture of five methoxyphenyl-disubstituted cyclobutanes found, together with *cis*-anethole. When an excited *trans*-anethole molecule interacts with one in the ground state, a cycloaddition leads to dimers 5 (*anti* head-to-head) and 7 (*syn* head-to-head) in Fig.2. For dimers 3, 4 and 6, *cis*-anethole reacted, which was itself formed from *trans*-anethole by photoisomerization. The abundance of dimers 5 and 7 is much higher than of those involving a *cis*-anethole as reaction partner (Castro et al., 2010).

Citral is a mixture of the isomers geranial (= *trans*-citral, substance 1 in Fig.3) and neral (= *cis*-citral, substance 2 in Fig.3) and one of the most important components of lemon EO, as it conveys the characteristic lemon-like odor. Iwanami et al. (1997) produced a lemon

flavor containing lemon oil and irradiated it with UV for 4 days at 30°C under nitrogen atmosphere to block oxidation reactions. The photodegradation led to the photoproducts shown in Fig.3: photocitral A (3), epiphotocitral A (4), photocitral B (5), 2-(3-methyl-2-cyclopenten-1-yl)-2-methylpropionaldehyde (6), *trans*-1,3,3-trimethylbicyclo[3.1.0]hexane-1-carboxaldehyde (7), *cis*-1,3,3-trimethylbicyclo[3.1.0]hexane-1-carboxaldehyde (8), (1,2,2-trimethyl-3-cyclopenten-1-yl)acetaldehyde (9), and α-campholenealdehyde (10). In the case of oxygen availability during irradiation, citral yields other transformation products, as different reactions are feasible in the presence of oxygen (see Chapter 2.2).

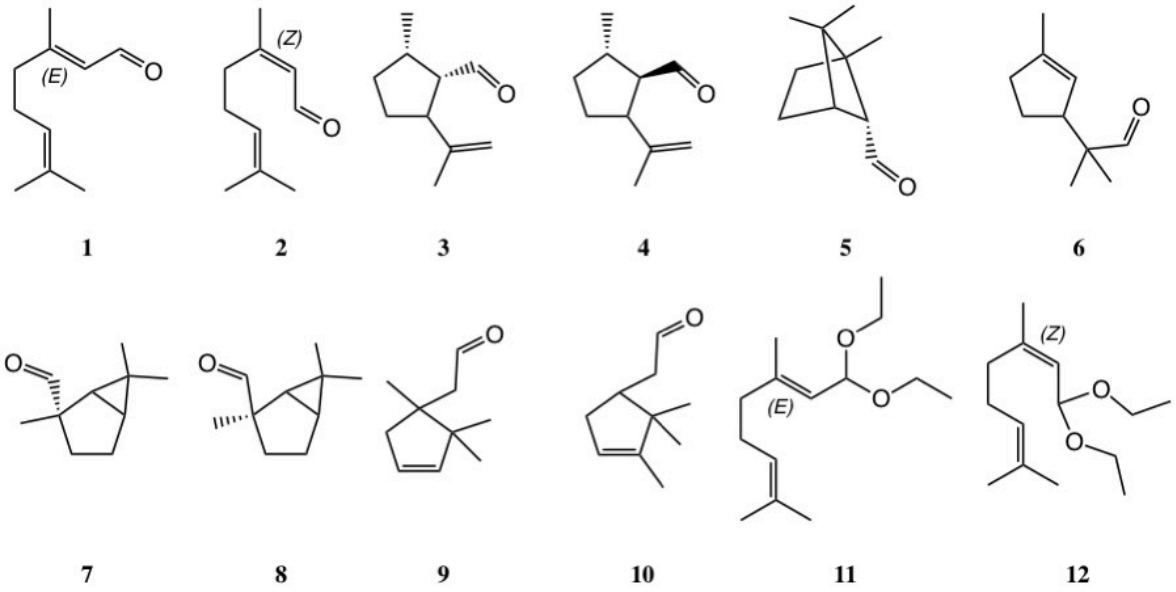

Figure 3: Photoreaction products of citral (adapted and newly drawn from Iwanami et al., 1997)

The formation of 6 requires a formyl 1,3-migration and is a newly identified photoreaction product. Diethyl acetals 11 and 12 were not obtained in the dark under these conditions, but might be formed by the hydrogen abstraction reaction of excited citral. Limonene, terpinolene and nonanal decreased in amount, whereas p-cymene increased. Other constituents, like for example citronellal, linalool, sesquiterpene hydrocarbons, and terpineols, were only insignificantly changed. The fresh, sweet, and characteristically lemon-like odor decreased and a dusty odor became predominant, which is mostly ascribable to compound 6 (Iwanami et al., 1997).

Krupa et al. (2012) investigated the isomerization reactions of **eugenol and isoeugenol** induced by tuneable UV laser light. They found that they could prompt photoisomerization in isoeugenol, which contains an asymmetrically substituted exocyclic C=C bond, by irradiation at different wavelengths ranging from 310-298nm. The *E*- and *Z*- isomers and their only practically significant and most stable rotamers are depicted in Fig.4.

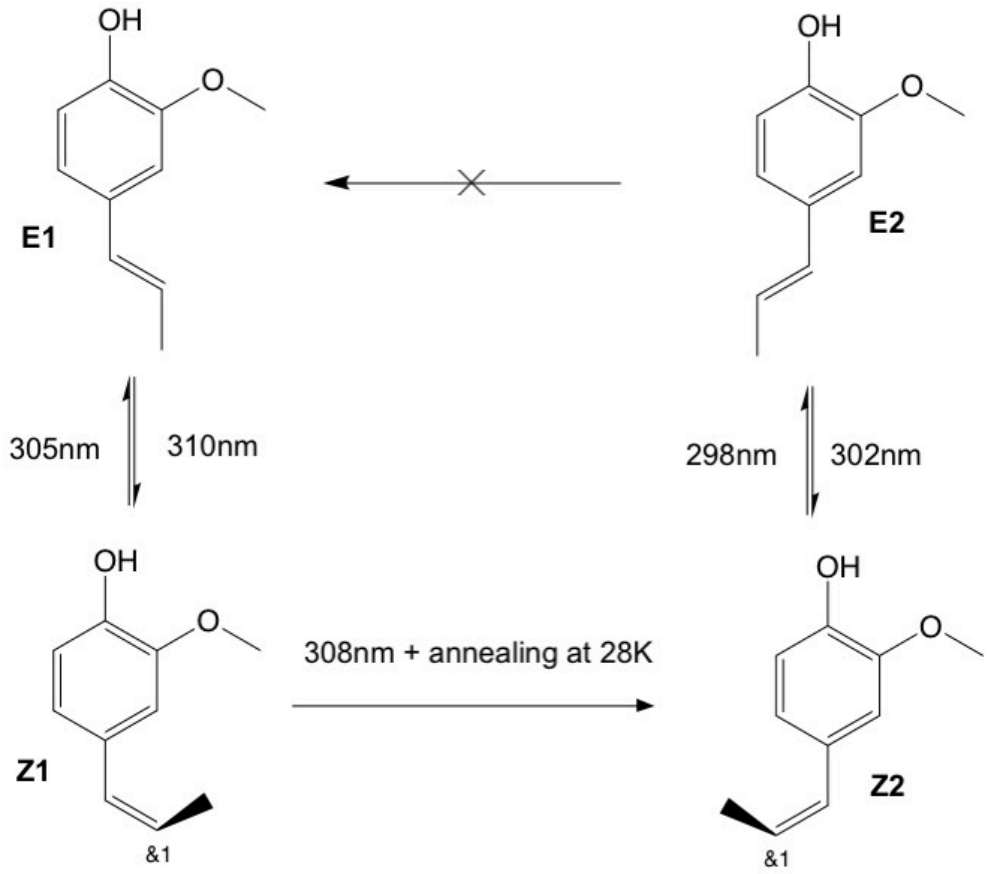

Figure 4: Interconversion reactions of eugenol (adapted and newly drawn from Krupa et al., 2012)

For the conversion of *E1* to *Z1*, a wavelength of 310nm was applied. This reaction could be reversed by irradiation at λ=305nm. These back and forth reactions between *E1* and *Z1* came to a halt at wavelengths of 306-308nm, suggesting a photoequilibrium. Irradiation at λ=302nm resulted in a conversion of *E2* to *Z2*. A partial back-transformation could be prompted with λ=298nm, but the spectral changes observed were not as pronounced as for the other interconversions. No changes in the infrared spectrum occurred following the annealing of freshly deposited isoeugenol on Ar matrices up to 30 K, which reveals that

the applied temperature and therefore energy does not suffice for *E2* to *E1* transformations. In an additional study combining UV irradiation and matrix isolation technique, isoeugenol on an Ar matrix was first irradiated at λ=308nm to increase the amount of *Z1*, followed by annealing of the matrix in steps of 2K starting at 15K. At 28K (= -245.15°C), the *Z1* bands decreased and *Z2* formed. When *Z1* and *Z2* forms reached equal amounts, the thermally induced partial reaction stagnated, despite further increase of temperature, suggesting they are isoenergetic. The *Z1* and *Z2* forms only occur in populations of about 4-5% each, since they are the energetically less convenient forms.

Besides isoeugenol, Krupa et al. (2012) also subjected eugenol to the same irradiation series, but came to the conclusion that no conformational changes were induced. However, aside from interconversions, they also studied photolysis of both eugenol and isoeugenol (see Fig.5) using narrow band UV irradiation technique. The reaction pathway is very similar for both molecules; therefore, it is illustrated on the example of eugenol. An H-atom shift from the OH-group was found to be the primary photochemical process for either of the compounds.

This hydrogen atom shift had a lower threshold energy in isoeugenol than eugenol (308nm vs. 285nm). In both cases, it resulted in the generation of two types of long-chain conjugated ketenes, depending on where the hydrogen atom repositioned itself on the ring. In another step of reaction, decarbonylation of the ketenes took place (Krupa et al., 2012).

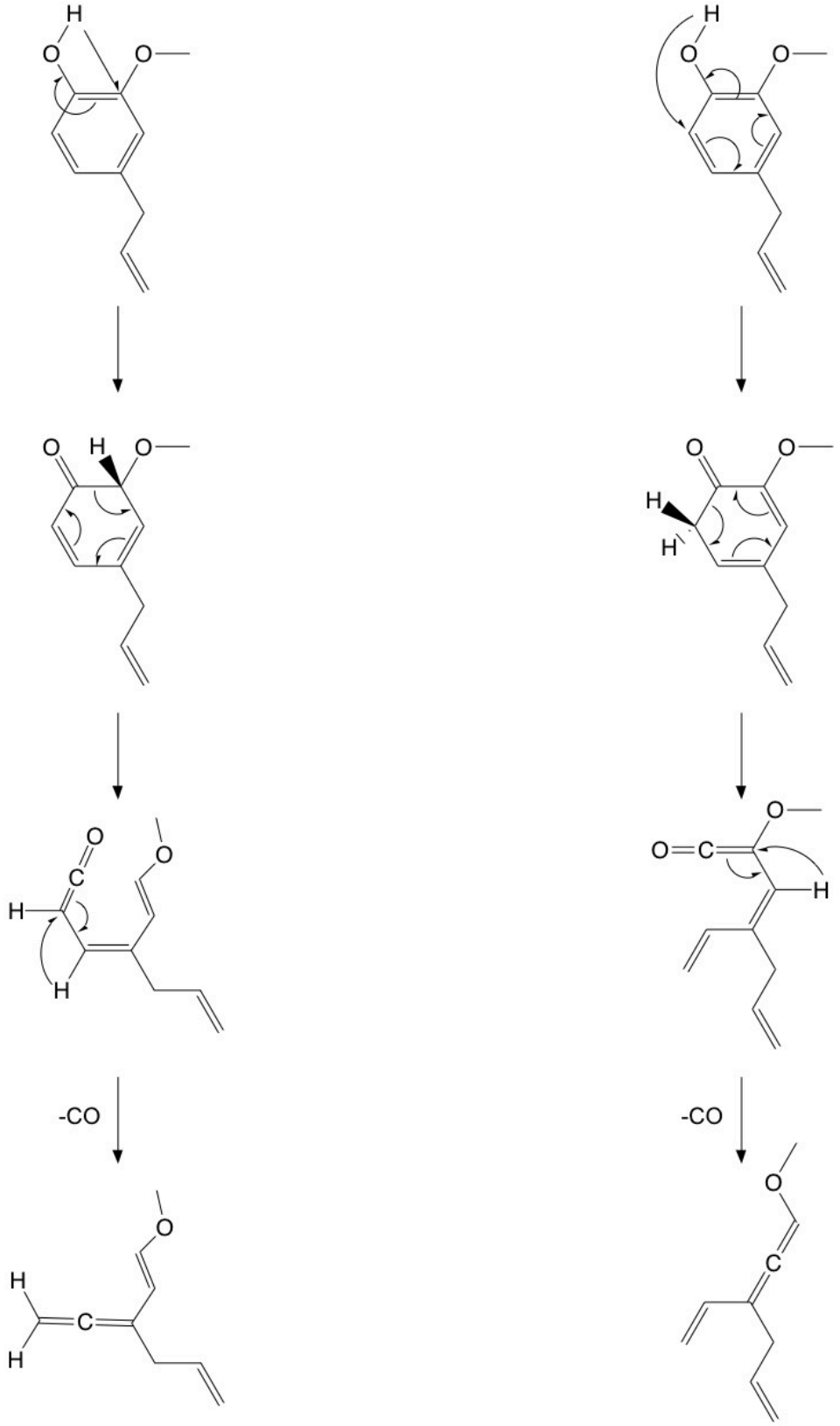

Figure 5: Photolysis pathways of eugenol (adapted and newly drawn from Krupa et al., 2012)

2.2. Photoxidation and –epoxidation reactions

Mori and Iwahashi (2016) studied the formation of radicals by oxidation of some EOs by measuring the electron spin resonance (ESR) spectra of reaction mixtures of flavin mononucleotide (FMN, an endogenous photosensitizer), EO, acetonitrile, phosphate buffer, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and $FeSO_4(NH_4)_2SO_4$, irradiated with visible light of 436nm. **Geraniol**, being its major constituent, gave similar results to palmarosa EO. In the reaction mixture with geraniol, a new radical, 4-POBN/5-hydroxy-3-methyl-3-pentenyl radical, was identified. Results showed that without light, Fe^{2+} or FMN, respectively, no reaction occurred. The authors proposed a possible reaction pathway, which is shown in Fig.6.

Supposedly, the irradiation with visible light generates the excited singlet state of FMN, ^{1}(FMN)*, which is then transformed by intersystem crossing, resulting in the excited triplet state, ^{3}(FMN)*. Subsequently, ^{3}(FMN)* probably reacts with triplet oxygen $^{3}O_2$, thereby forms $^{1}O_2$, which in turn produces 3,7-dimethyl-6-hydroperoxy-2,7-octadienol (compound 2 in Fig.6) following the singlet oxygen *ene*-reaction with geraniol (compound 1 in Fig.6). Considering that the reaction does not occur without the ferrous ions, Mori and Iwahashi presume that they catalyze the cleavage of this newly formed compound via β-scission of the alkoxy radical intermediate (compound 3 in Fig.6), yielding the newfound radical. Under the same reaction conditions, geranium, clary sage, lavender, petitgrain, and bergamot EO also gave strong ESR signals, a fact which the authors attribute to the autoxidation potential of their constituents, such as geraniol, limonene and linalool (Mori/Iwahashi, 2016).

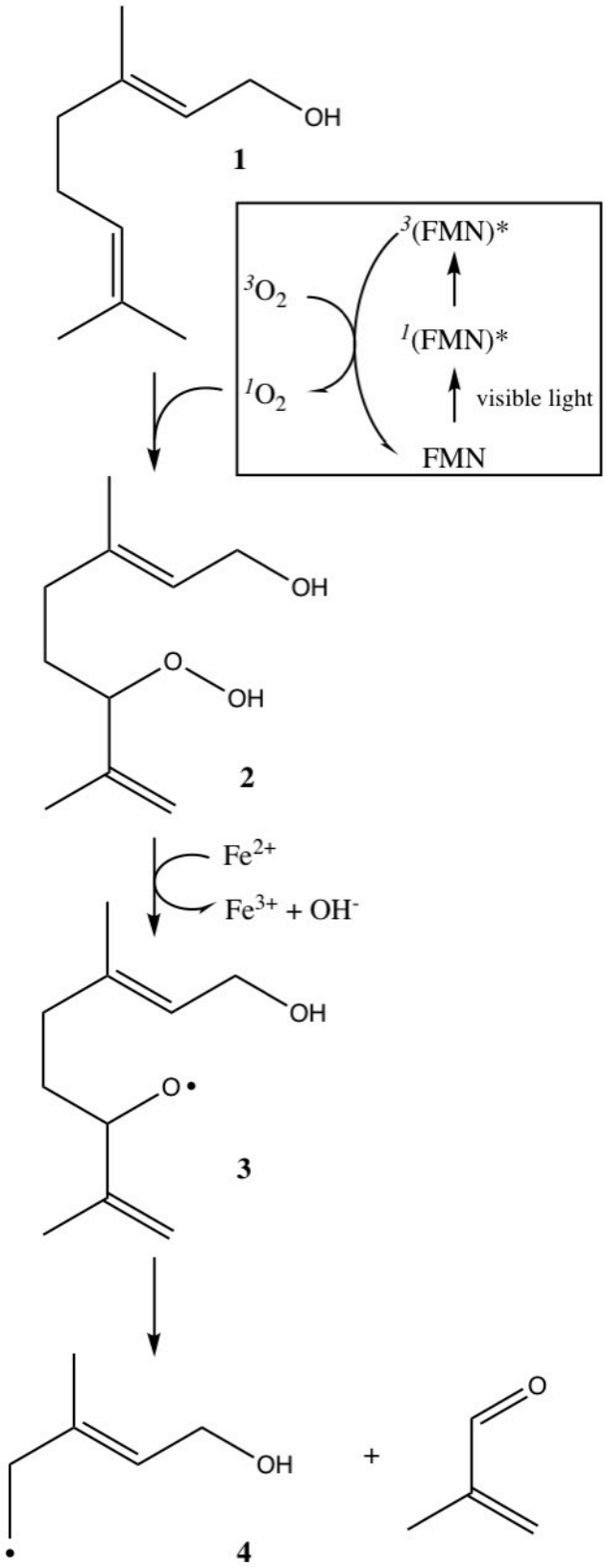

Figure 6: Geraniol radical formation (adapted and newly drawn from Mori/Iwahashi, 2016)

Ziegler et al. (1991) studied deterioration products and processes of **sweet orange EO** (*Citrus sinensis* L. Osbeck) under simulated aging conditions, exposing an aqueous acidic orange oil emulsion to UV light at room temperature. The subsequent GC/MS analysis disclosed an increase in carvone, isopulegol, isomers of carveol, the limonene and linalool oxides, as well as a significant decrease in neral, geranial and citronellal. The authors also identified p-mentha-l,8-dien-4-ol, α-cyclocitral, photocitral A, iso(iso)pulegol, carvone, camphor, menthone, isomenthone, isomers of p-mentha-l(7),8-dien-2-ol and isopiperitenol as newly formed secondary constituents (Ziegler et al., 1991).

The effects of different storage conditions on **rosemary EO** were investigated by Irmak et al. (2010), who stored the EO at 4°C in the dark or at room temperature in indirect daylight for 14 weeks. Considering that they did not only use different light conditions but also varied the temperature, it is difficult to say which factor was crucial, but under the daylight conditions, substantial chemical transformations occurred, whilst the storage in darkness and low temperature did not have much of an impact. The authors studied the change in total phenolics content and antioxidant properties of the rosemary EO following storage periods of 0, 2, 4, 8, and 14 weeks. The rosemary EO samples showed high antioxidant activity when fresh, largely preventing the bleaching of beta-carotene in the assay. But this capability, and the total phenolics content likewise, diminished after the storage time, especially in the extracts stored in light. The GC-MS peaks of *trans*-caryophyllene and squalene disappeared completely in one of the samples stored under indirect daylight, and in another there was a reduction of 38% for linalool, 24% for limonene, and 44% for *trans*-caryophyllene (Irmak et al., 2010).

Li et al. (2016) investigated the transformations occurring during UV and air exposure in the **EO of white guanxi honey pummelo** (*Citrus grandis* (L.) Osbeck, Rutaceae), a citrus variety from southeast Asia. The main constituents of pummelo are (+)-limonene and β-myrcene, germacrene D, geranial, neral, β-pinene, linalool, sabinene, and α-pinene (Sun et al., 2014). EO was mechanically pressed from the fruit and one sample was irradiated with UV light for 40h while being exposed to air at approximately 25°C. In order to identify degradation mechanisms, single standard aldehydes without solvent dilution, i.e., octanal (99%), nonanal (96%), citronellal (96%), decanal (95%), citral (97%, mixture of *trans*-

citral and *cis*-citral), perilla aldehyde (92%), dodecanal (95%), and dodecenal (93%), were subjected to air exposure, UV irradiation, or a combination of both (Li et al., 2016).

After UV light and air cotreatment, the concentrations of octanal, nonanal, decanal, dodecanal, dodecenal, perilla aldehyde, *trans*-citral, and *cis*-citral in the pummelo EO decreased by 13.8, 28.3, 40.5, 37.8, 85.4, 33.9, 85.6, and 82.1% ($p < 0.05$), respectively. The only aldehyde not sticking to this pattern was citronellal, which in contrast increased by 84.6%, probably due to precursors existing in the EO. The other aliphatic aldehydes were found to be transformed to their organic acids after the combined exposure to air and UV light, a process in which the UV light grants the energy necessary and the air provides the oxygen for the oxidative reaction (Li et al., 2016). The authors suggested a possible reaction pathway for the aliphatic aldehydes and citral in their article (see Fig.7).

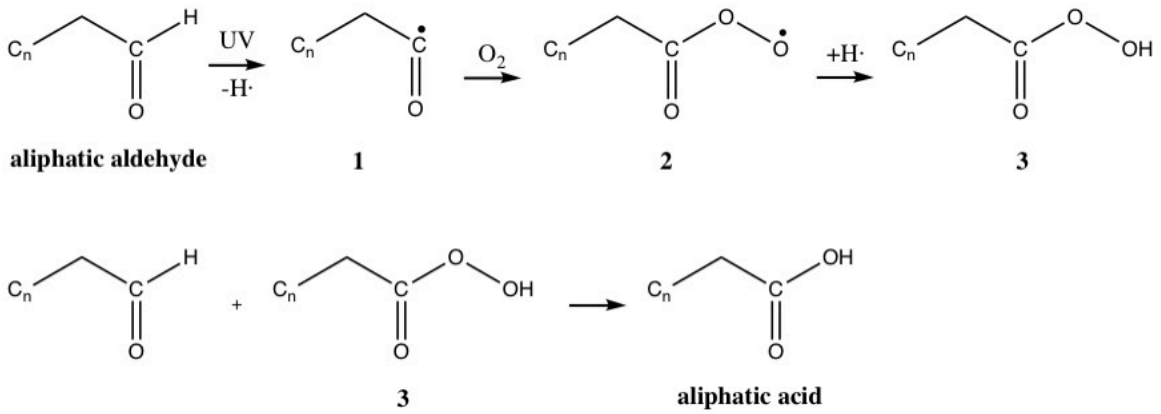

Figure 7: Aliphatic aldehyde photoxidation, n=6 (octanal), 7 (nonanal), 8 (decanal), 10 (dodecanal) (adapted and newly drawn from Li et al., 2016)

The reaction process seems to be the same for all aliphatic aldehydes, starting with the UV-induced loss of a hydrogen radical by the carbon at position 1, generating a free radical (compound 1 in Fig.7), followed by an attack by oxygen resulting in a peroxide radical (compound 2 in Fig.7), which then associates with a hydrogen radical and subsequently attacks and oxidizes an original aldehyde molecule, yielding the corresponding aliphatic acid. Neither air nor oxygen alone could trigger the reactions of the aliphatic aldehydes in the study (Li et al., 2016).

***trans/cis*-Citral** was transformed to cyclocitral under exposure to UV light, with or without oxygen availability. Under cotreatment of air and irradiation, citral reacted to form geranic acid and neric acid, in contrast to the study of Schieberle/Grosch (1989), who found

citral to be transformed to *p*-methylacetophenone after 4 days in 5% citric acid at 40°C, suggesting that citral undergoes different reaction pathways under different conditions (Li et al., 2016). The formation of cyclocitral is explained by the authors as a succession of steps, starting once more with the loss of a hydrogen radical at the α-position carbon of the carbon at position 3. After a recombination of carbon-carbon double-bonds, the position 2 carbon attacks the double-bond in position 7, resulting in a cyclic free radical (compound 3 in Fig.8), which then associates with a hydrogen radical to form an intermediate (compound 4 in Fig.8). Another hydrogen radical loss and double-bond recombination occur, until cyclocitral is formed by hydrogen radical uptake.

Another putative reaction pathway leads to the citral acids, also starting with the loss of a hydrogen radical at position 1, generating a free radical (compound 6 in Fig.8) which is subsequently attacked by oxygen, forming a peroxide radical (compound 7 in Fig.8). After forming another intermediate by associating with a hydrogen radical, this compound oxidizes *trans/cis*-citral, accordingly yielding the corresponding citral acid. The transformation pathways are depicted for *cis*-citral in Fig.8; for *trans*-citral the mechanism works analogously, ultimately generating also cyclocitral, but geranic acid instead of neric acid.

This change in constituents is followed by a change in odor of pummelo EO. After the treatment with light and oxygen, the minty, herbaceous and lemon odors decrease, and the oily odor intensifies, due to the concentration reduction of β-myrcene, (+)-limonene and aldehydes, i.e. octanal, decanal, *cis*-citral, *trans*-citral, and dodecenal (Li et al., 2016).

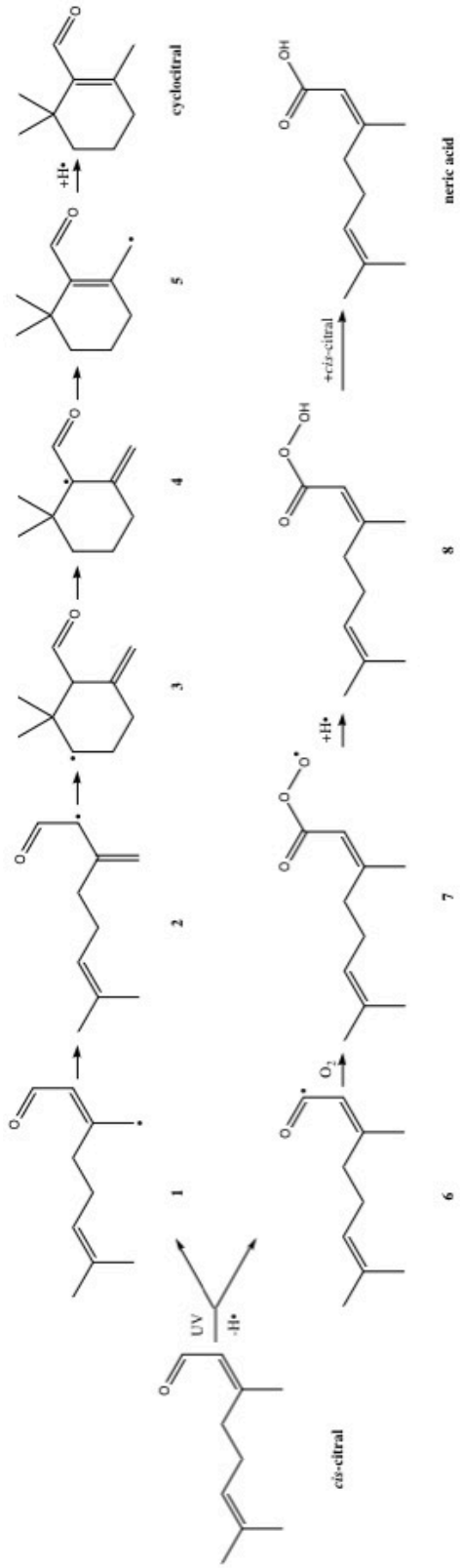

Figure 8: Citral phototransformations (adapted and newly drawn from Li et al., 2016)

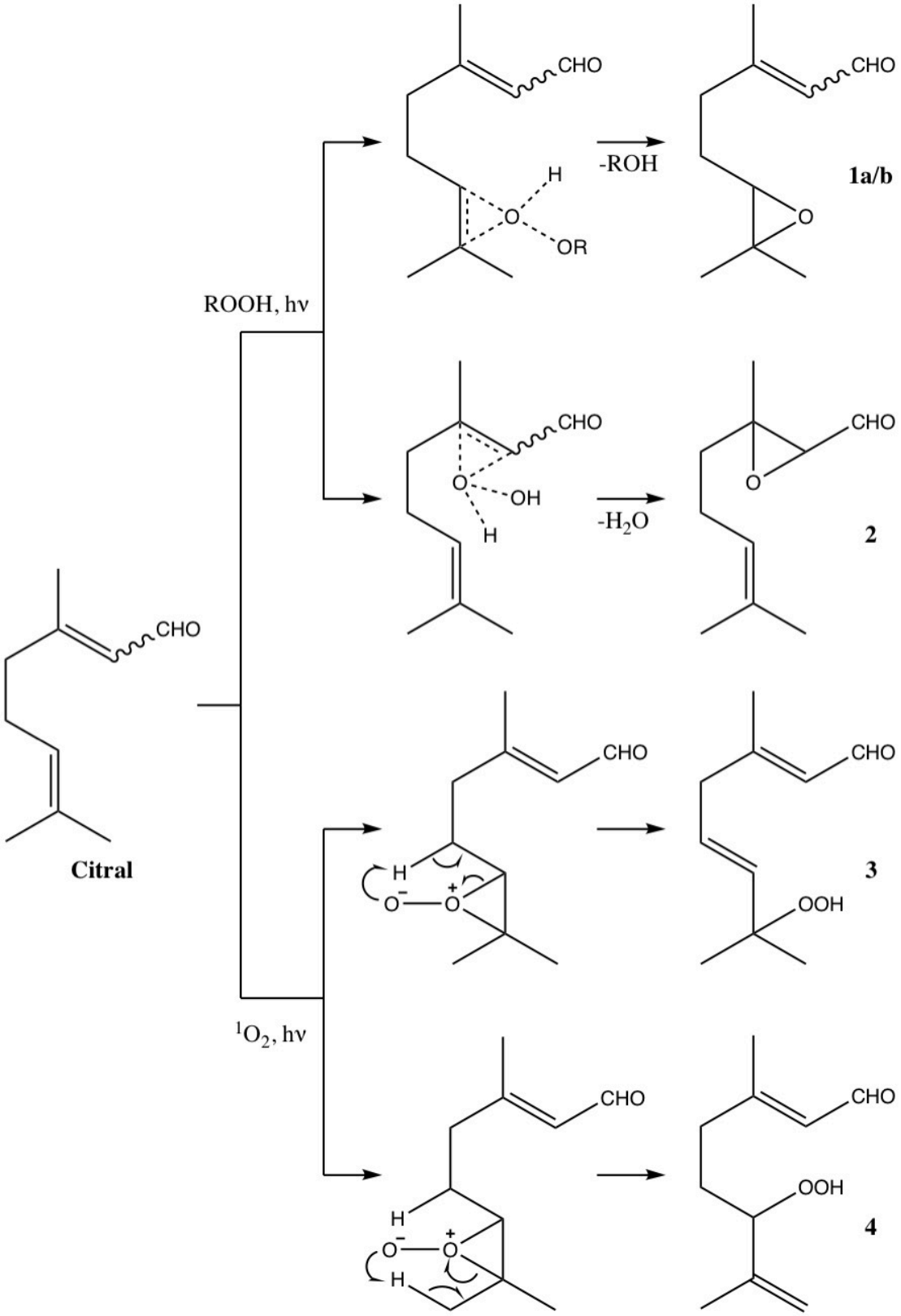

Figure 9: Photoepoxidation reactions of citral, R = H, 3-$ClC_6H_4CO_3H$ (adapted and newly drawn from Elgendy/Khayyat, 2008)

Interestingly, citral seems to undergo very different reactions when brought into contact with hydrogen peroxide instead of molecular oxygen during irradiation. Elgendy/Khayyat (2008) studied the photoxidations of citral, pulegone and camphene under irradiation with a sodium lamp under different conditions. In one reaction 30% hydrogen peroxide was added, in another 80% m-chloroperoxybenzoic acid (3-$ClC_6H_4CO_3H$) as an oxidant and in yet another a singlet oxygen photosensitizer, i.e. tetraphenylporphyrin (TPP) or Rose Bengal or chlorophyll. The mixture of citral and hydrogen peroxide in ethanol was subjected to irradiation for 55 hours, and the reaction yielded (2E,Z)-5-(3,3-dimethyloxiran-2-yl)-3-methylpent-2-enal (compounds 1a/b in Fig.9) and 3-methyl-3-[(3E)-4-methylpent-3-en-1-yl]oxirane-2-carbaldehyde (compound 2 in Fig.9). Oxidation with the acid in chloroform at room temperature under nitrogen generated the same two isomers 1a and 1b, but no others products were detectable. Interestingly, the photosensitized reaction gave a mixture of (2E,5E)-7-hydroperoxy-3,7-dimethylocta-2,5-dienal (compound 3 in Fig.9) and (2E)-6-hydroperoxy-3,7-dimethylocta-2,7-dienal (compound 4 in Fig.9). The highest yield was achieved with TPP as a sensitizer. Structure 3 was found to have DNA damaging effects in the study (Elgendy/Khayyat, 2008).

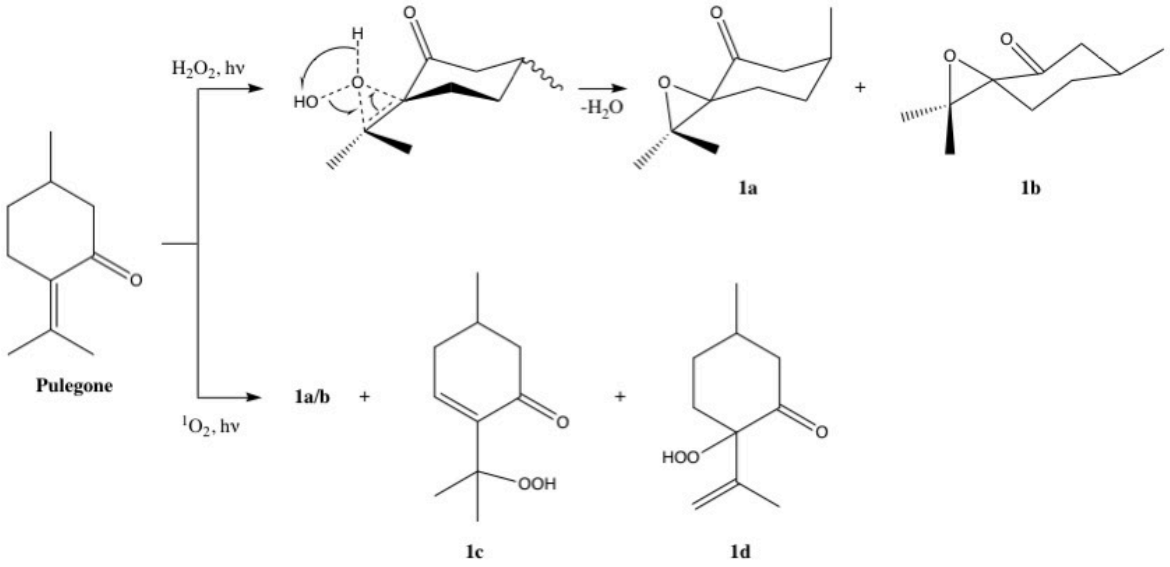

Figure 10: Photoepoxidation reactions of pulegone (adapted and newly drawn from Elgendy/Khayyat, 2008)

Pulegone is another natural monoterpene found in the EOs of many different *Mentha* species and was isolated for the study of Elgendy/Khayyat (2008) from the leaves of *Mentha*

pulegium (Lamiaceae). The photoreaction products of pulegone with 30% hydrogen peroxide in ethanol were compounds 1a and 1b, a mixture of isomers with different mutual stereochemical orientations at the oxirane ring and the 7-methyl group, as depicted in Fig.10. In relation to the cyclohexane ring, the addition of hydrogen peroxide can occur on either side of the exocyclic double-bond, therefore two isomers are generated after the loss of H_2O by the intermediate oxirane. In the resulting products, the position of the methyl group on C7 and the oxirane oxygen atom are relative to each other in *trans*-position for compound 1a and in *cis*-position for compound 1b. The reaction in the presence of a photosensitizer like tetraphenylporphyrin, Rose Bengal or chlorophyll, interestingly, produced (besides 1a/b) two additional compounds: 2-(1-hydroperoxy-1-methylethyl)-5-methylcyclohex-2-en-1-one (compound 2 in Fig.10), and 2-hydroperoxy-2-isopropenyl-5-methylcyclohexan-1-one (compound 3 in Fig.10). This oxygenation most likely involves the stabilization of a peroxirane transition state along two different pathways, analogue to the generation of products 3 and 4 in the photosensitized reaction of citral. Compound 2 was found to be genotoxic in a DNA damage assay by the authors (Elgendy/Khayyat, 2008).

The photoxidation of **camphene**, another monoterpene found for example in the EO of camphor, lemongrass and ginger, was also studied by Elgendy/Khayyat (2008). The reaction with 30% hydrogen peroxide produced a mixture of *endo*- and *exo*-isomers of 3,3-dimethylspiro[bicyclo[2.2.1]-heptane-2,2′-oxirane] (compounds 1a and b in Fig.11) and camphor (compound 2 in Fig.11) in approximately 15% yield, while its thermal oxidation with m-chloroperoxybenzoic acid gave only the two former products. The generation of camphor in this setting is described as “unusual” by the authors, but nevertheless they propose a possible formation pathway featuring a photoinitiated rearrangement of camphene followed by the attack of hydrogen peroxide (see Fig.11).

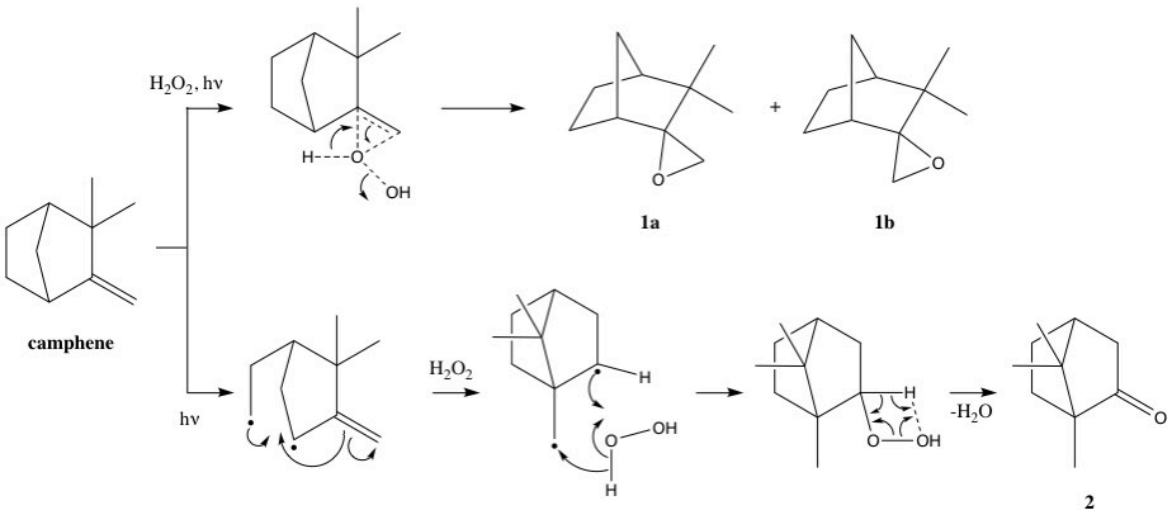

Figure 11: Photoxidation reactions of camphene (adapted and newly drawn from Elgendy/Khayyat, 2008)

Linalyl acetate is an acyclic monoterpene very commonly used in floral scents and poses one of the main constituents of lavender (*Lavandula angustifolia* Mill., Lamiaceae) EO. Khayyat (2018) extracted linalyl acetate from lavender EO and exposed it to 30% hydrogen peroxide and irradiation from a sodium lamp in a nitrogen atmosphere at 0°C in ethanolic medium. The main reaction products were 6,7-epoxy-3,7-dimethyl-1-octene-3-yl acetate (compound 1 in Fig.12) and 1,2-epoxy-3,7-dimethyl-6-octene-3-yl acetate (compound 2 in Fig.12), which are basically two different epoxides of linalyl acetate depending on which double-bond has been attacked by hydrogen peroxide. The reaction with m-chloroperbenzoic acid instead of hydrogen peroxide yielded only compound 1. When tetraphenylporphyrin (TPP) was used as a photosensitizer, a mixture of 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-yl acetate (compound 3 in Fig.12) and 6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-yl acetate (compound 4 in Fig.12) was produced, whereas hematoporphyrin (HP) as a sensitizer only gave compound 3 as a product. The reaction pathways proposed by the author are depicted in Fig.12 (Khayyat, 2018).

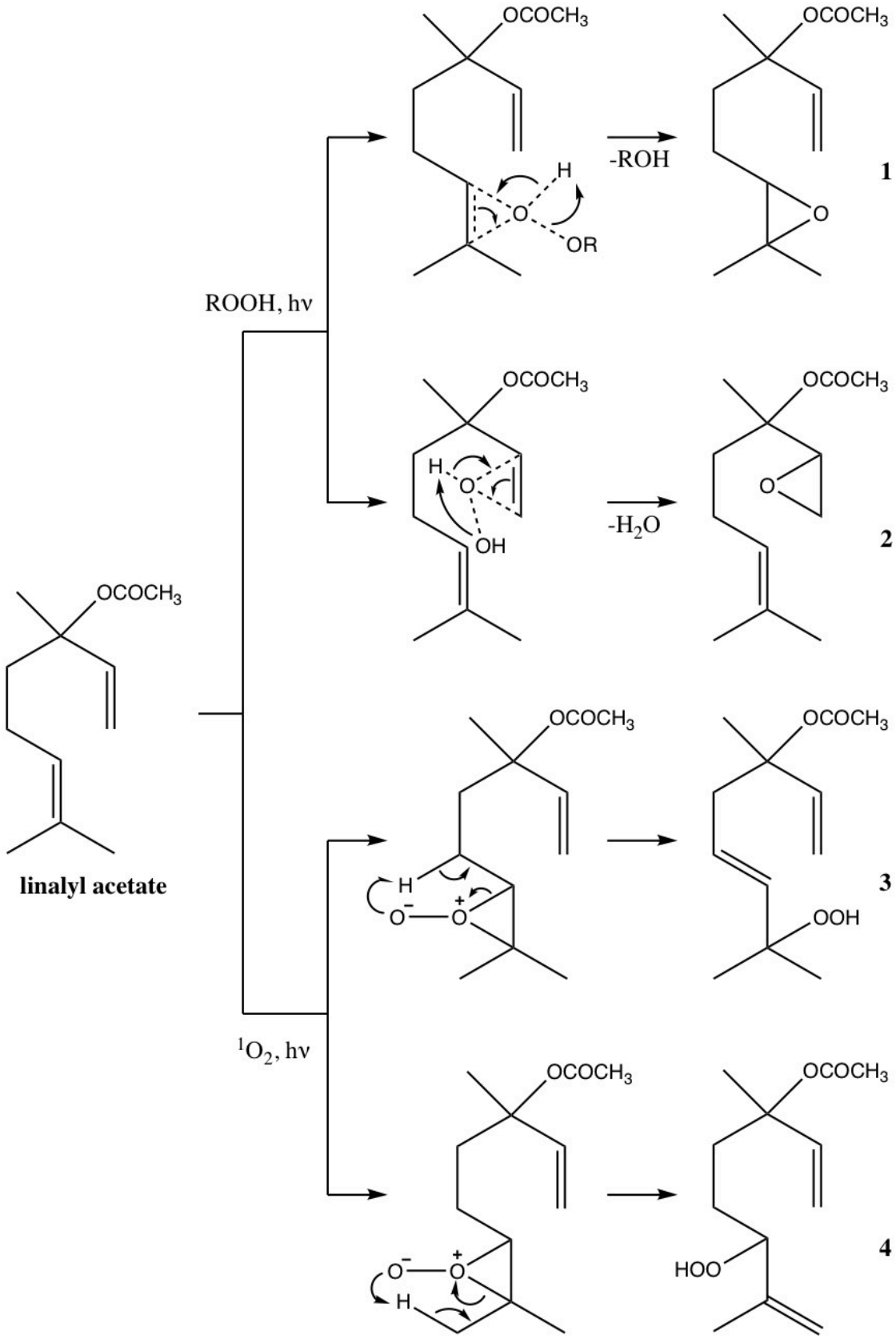

Figure 12: Photoepoxidation reactions of linalyl acetate, R = H, 3-ClC6H4CO3H (adapted and newly drawn from Khayyat, 2018)

Saffron is, amongst other uses, a spice consisting of the dried stigmas of *Crocus sativus* L. (saffron, Iridaceae) and one of the most expensive spices in the world (Raghavan, 2006). The most abundant constituent of saffron EO, **safranal**, is mainly responsible for the typical saffron aroma, and was found to elicit many effects on the central nervous system, such as antidepressant, anticonvulsive and hypnotic effects, and many more (Rezaee/Hosseinzadeh, 2013). Khayyat/Elgendy studied safranal epoxidation, adding 30% hydrogen peroxide and subduing the mixture to 50 hours of irradiation with a sodium lamp under nitrogen

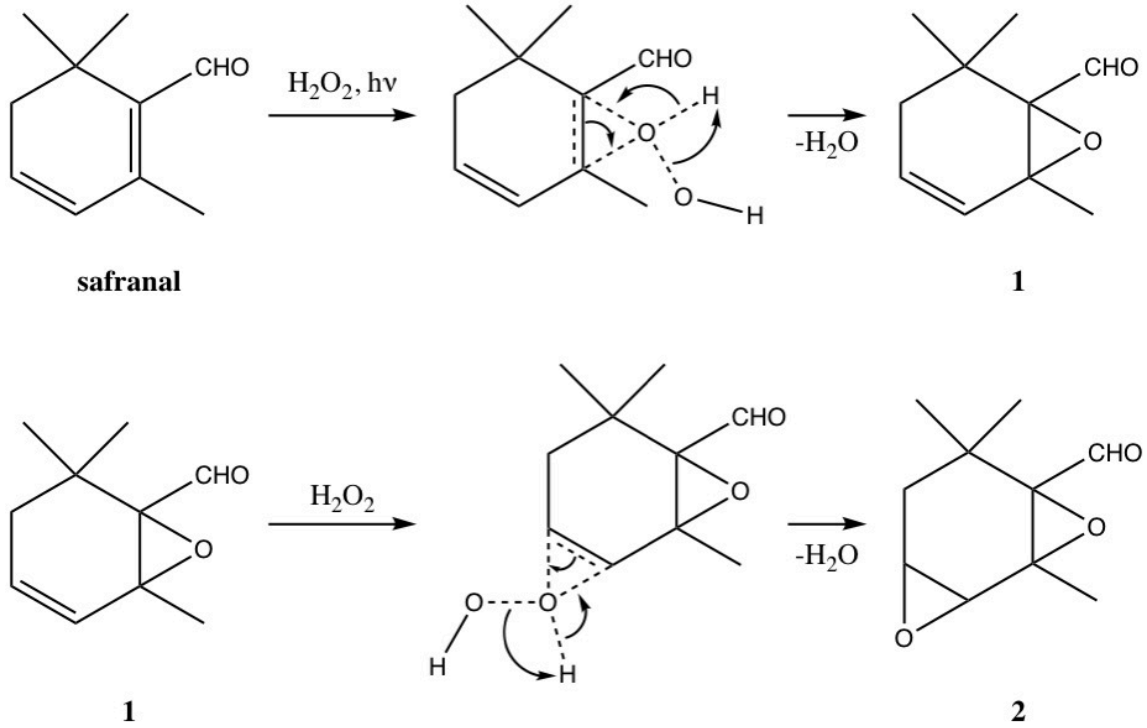

Figure 13: Photoepoxidation reactions of safranal (adapted and newly drawn from Khayyat/Elgendy, 2018)

atmosphere. The photochemical reaction resulted in 2,2,6-trimethyl-7-oxabicyclo[4.1.0]-hept-4-ene-1-carbaldehyde (compound 1 in Fig.13) and diepoxy derivative 2,5,5-trimethyl-3,8-dioxa-tricyclo[5.1.0.02,4]octane-4-carbaldehyde (compound 2 in Fig.13), in yields of 65 and 35% respectively. The proposed reaction pathways are shown in Fig.13. Subsequent analysis of the antibacterial activity of the educt and products proved that the monoepoxy and diepoxy derivatives of safranal possess an increased effect against methicillin resistant *Staphylococcus aureus* (MRSA).

Khayyat/Sameeh (2018) investigated the photoxidation of geranyl acetate, which is an acyclic monoterpene occurring in the volatile oils of many plant species, such as eucalyptus, cypress and origanum, for instance. When brought into contact with 30% hydrogen peroxide in ethanolic medium and irradiated with a sodium lamp for 15 hours, geranyl acetate was oxidized at both double-bonds and yielded a diepoxy product, 3-(2-(3,3-dimethyloxiran-2-yl)ethyl)-3-methyloxiran-2-yl) methyl acetate. On the other hand, the reaction in presence of a photosensitizer resulted in a photooxygenation with three different mono- and dihydroperoxide derivatives, acetic acid 2,6-bis-hydroperoxy-7-methyl-3-methylene-oct-7-enyl-ester, acetic acid 7-hydroperoxy-3,7-dimethyl-octa-2,5-dienyl ester and acetic acid 3-hydroperoxy-7-methyl-3,7-dimethyl-octa-1,6-dienyl ester (Khayyat/Sameeh, 2018).

2.3. Polymerization reactions

Some constituents of EOs polymerize when exposed to light and air. Khayyat (2013) studied the photopolymerization of ***trans*-cinnamaldehyde, eugenol and safrole** by allowing them to react in chloroform at room temperature with oxygen availability and during irradiation with a sodium lamp. *trans*-Cinnamaldehyde is an aromatic aldehyde, which occurs naturally in the bark of species of the genus *Cinnamomum* (Lauraceae) and poses the main origin of the characteristic cinnamon aroma. Eugenol is the main component of the EO of *Eugenia caryophyllus* (Spreng., Myrtaceae), and safrole, on the other hand, is the major constituent of the EO extracted from sassafras (see below). All three produced their corresponding dimers: cinnamaldehyde gave 4,6-diphenyl-1,2-dioxane-3,5-dicarboxaldehyde (compound 1 in Fig.14), eugenol gave 4-4'(cyclobutane-1,3-diyl-bis(methylene)bis-(2-methoxyphenol) (compound 2 in Fig.14) and safrole gave 3,6-bis(benzo[d][1,3]dioxol-5-ylmethyl)-1,2-dioxane (compound 3 in Fig.14). The yields were 73%, 62% and 55%, respectively.

A probable reaction mechanism for both cinnamaldehyde and safrole is [2+2+2] cycloaddition following singlet oxygen attack on the double-bond of the side-chain for two molecules each, in both cases resulting in a dimer with a dioxane ring (see Fig.14). As for eugenol, this compound yielded a dimer with a cyclobutane ring after a [2+2] cycloaddition reaction (Khayyat, 2013).

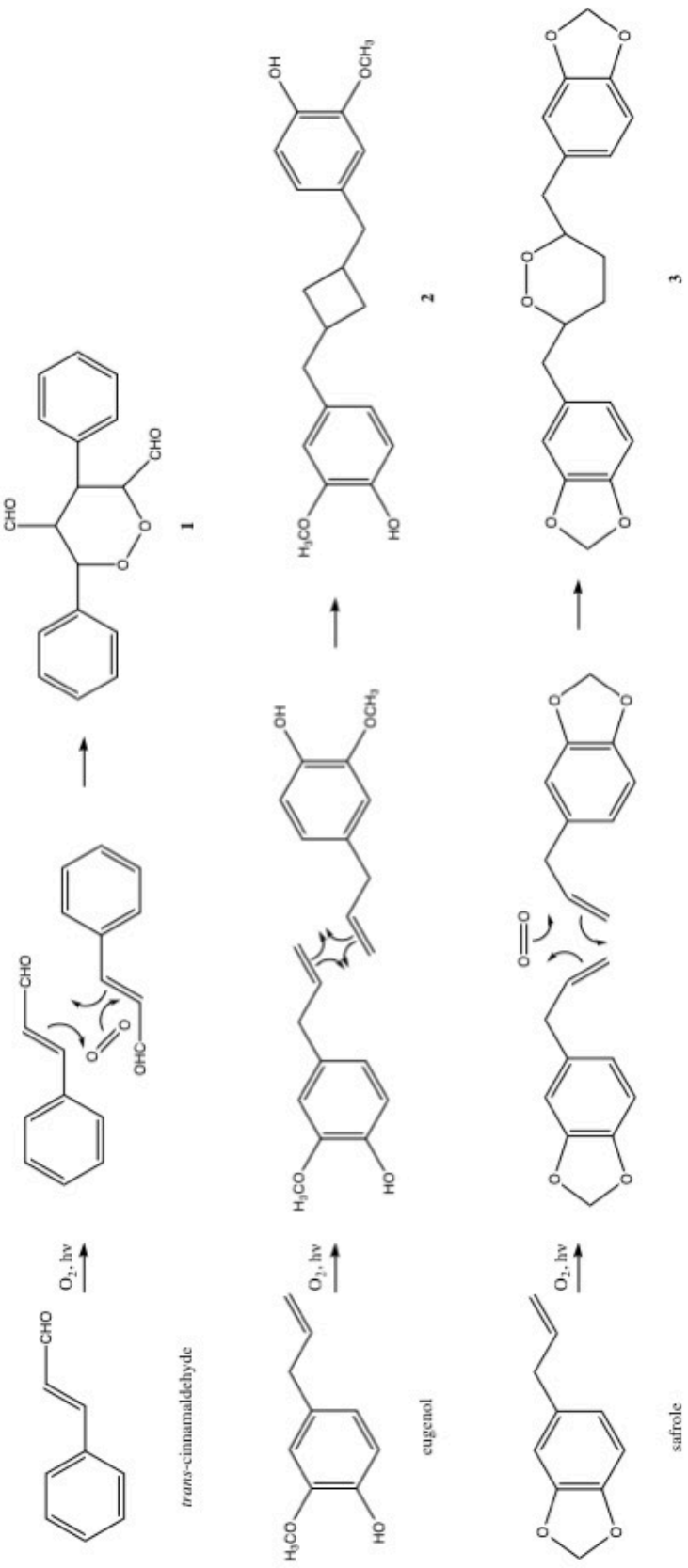

Figure 14: Photopolymerization of trans-cinnamaldehyde, eugenol and safrole (adapted and newly drawn from Khayyat, 2013)

The resulting dimers were tested for their antifungal activity against *Candida albicans*, a very common form of candida yeast which naturally inhabits several moist and warm cavities of the human body but can also cause skin and mucosal infections, in comparison to their respective monomers. The results indicated that the dimers elicit a stronger antifungal effect than their monomer precursors (Khayyat, 2013).

Isoeugenol was also found to undergo dimerization under irradiation with UV light. Chiang/Li (1978) subjected isoeugenol in actone to the light of a high pressure mercury lamp, triggering a photoreaction that results in two different dimers of isoeugenol: diisoeugenol (= 1-ethyl-3-(4'-hydroxy-3'-methoxyphenyl)-6-methoxy-2-methyl-5-indanol) and dehydrodiisoeugenol (= 4-[2',3'-dihydro-7'-methoxy-3'-methyl-5'-(1''-propenyl)-2'-benzofuranyl]-2-methoxy-phenol) (Chiang/Li, 1978).

Dimerization of ***trans*-anethole** was described by Lewis/Kojima (1988) as a cycloaddition of a cation radical and a neutral, which yields a mixture of *syn* and *anti* head-to-head dimers. The reaction products are depicted in Fig.2 (see above), together with the isomerization products, as irradiation of *trans*-anethole in acetonitrile or toluene gave *cis*-anethole as well as the various dimers, the concentrations of the latter depending on whether a sensitizer was used (Lewis/Kojima, 1988; Castro et al. 2010).

2.4. Other reaction mechanisms

Moulin et al. (1995) used laser photolysis as an alternative to other separation techniques for purifying complex mixtures such as extracts and EOs by selectively eliminating non-desirable molecules. They monitored the destruction of the molecules by spectral changes and identified the photoproducts by gas chromatography and mass spectrometry. Salvia and bergamot EOs were diluted tenfold and subjected to a laser beam in order to purge the contained amount of toxic thujone and phototoxic bergapten, respectively.

Salvia EO was irradiated at 308nm, and **thujone** reacted to yield two major reaction products 1 and 2, which are depicted in Fig.15. The supposed driving force of the reaction is ring strain relief, and the process involves a Norrish type 1 cleavage, a loss of carbon monoxide and subsequent recombination of the radical fragments. Other molecules in the

EO, such as pinene, cineol and caryophyllene, were not affected by the laser photolysis, except for **camphor**, which is also a ketone with a very similar structure and absorption spectrum to thujone. The camphor degradation products 3 and 4 are shown in Fig.15, although it must be noted that they only posed a small percentage of the original amount of camphor, and the authors suggest that another new reaction product with a higher molecular weight was formed, probably due to rearrangement with EtOH (Moulin et al., 1995).

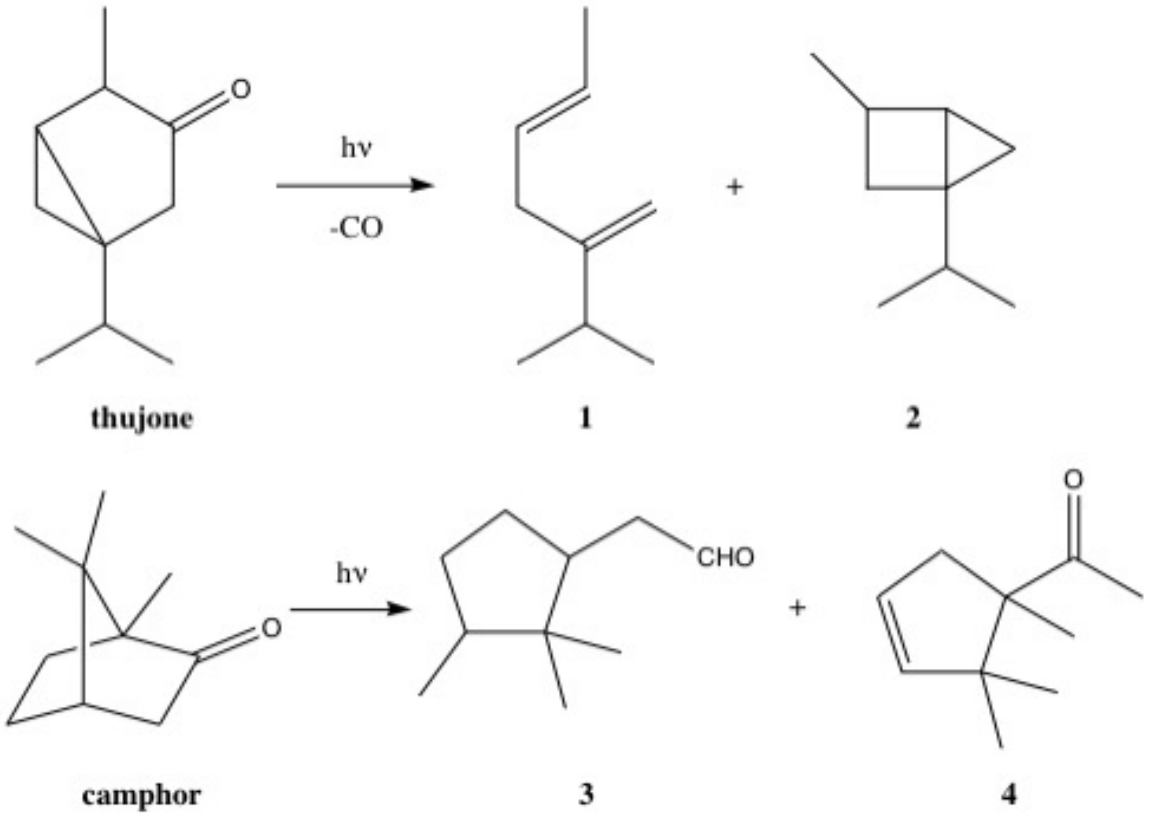

Figure 15: Thujone and camphor photolysis (adapted and newly drawn from Moulin et al., 1995)

Bergapten (= 5-methoxypsoralen) causes phototoxic reactions when skin treated with bergamot EO is exposed to UV light. The laser photolysis at 10 Hz by Moulin et al. (1995) eliminated 35% of the bergapten in the EO after 1 hour, and 60% after 2 hours of irradiation. Other constituents were not affected, and the process seems to be irreversible due to unchanging absorption spectra several days after the phototreatment (Moulin et al., 1995). In their study, the authors also aimed for the destruction of **safrole** in the oil of sassafras, which can be obtained from the roots of *Sassafras albidum* (Nutt., Lauraceae) or some members of the genus *Ocotea*, which are closely related. Furthermore, it can be sourced

from *Piper hispidinervum* (C.DC., Piperaceae), the EO of which also contains high levels of safrole (Rocha/Chau Ming, 1999). Interestingly, the trade of plant extracts rich in safrole is restricted in the European Union, due to the fact that safrole is a precursor in the production of MDMA (EMCDDA, 2016). Additionally, safrole is known to have carcinogenic effects. The sassafras EO was subdued to laser photolysis and with the help of chromatograms before and after the authors could verify the complete disappearance of the safrole peak. Instead, three new peaks appeared, which can be assumed to be those of short fragments of safrole, because of their short retention times and because laser photolysis generally creates smaller products in comparison to UV photoreactions, a fact attributed to the large difference in energy (Moulin et al., 1995).

Turek/Stintzing (2012) studied the impact of different storage conditions on EOs using the example of **rosemary, thyme, pine and lavender EO**. They simulated three different storage circumstances: storage A involved amber glass vials at room temperature in the dark, whereas the alternatives were clear glass vials submitted to cool white light simulating daylight for 24 hours per day at room temperature (23 ± 3°C, storage B) or elevated temperature (38 ± 3°C, storage C). The most striking transformation was observed in rosemary (*Rosmarinus officinalis* L., Lamiaceae) EO, where the amount of the monoterpene α-terpinene did not change under storage condition A, but decreased to under 10% of the original value at elevated temperature under light irradiation after mere 3 weeks. Both storage experiments under daylight conditions revealed accelerated degradation under irradiation. Phototransformations resulted in a noticeable decrease in α-terpinene and α-phellandrene together with an increase in p-cymene, camphor, 1,8-cineole and caryophyllene oxide, as well as some yet unidentified oxidation products.

The EO of thyme (*Thymus vulgaris* L., Lamiaceae) showed a distinctive stability under all conditions tested. Minor diminutions of β-myrcene, γ-terpinene, and α-terpinene could be detected in the elevated temperature of storage C, but also, to a lesser extent, in the dark. The high amount of radical-scavenging phenolic structures may be accountable for the resistance of thyme EO to degradation.

The EO of *Pinus sylvestris* L. (Pinaceae) showed a peculiar behavior: the highest peroxide values were obtained with storage in the dark, and moreover, the peroxide values

were lower at intensified storage conditions C than at room temperature. The authors propose a lability of the hydroperoxide intermediates at higher temperatures to explain these results. The amount of p-methylacetophenone, caryophyllene oxide, as well as a range of newly generated polar compounds increased significantly, whereas many other unidentified substances decomposed already within 4 to 8 weeks. In summary, pine EO seems more prone to oxidation than the former two EOs, as several constituents such as caryophyllene oxide experienced alterations even in the dark.

Lastly, Turek/Stintzing (2012) also investigated the EO of *Lavandula angustifolia* Mill. (Lamiaceae). Already after 4 weeks under daylight conditions (storage B) the peroxide value was higher than after 12 weeks of storage in the dark. However, similar to pine EO, storage under aggravated conditions (38°C, storage C) yielded lower peroxide values than at room temperature, again suggesting thermolabile hydroperoxy intermediates. Unfortunately, most compounds affected by the transformations were not identified by the authors, but it can be stated that imitated daylight conditions forced the total breakdown of an EO constituent and a remarkable decrease of another, while the formation of one structure turned out to be promoted by irradiation (Turek/Stintzing, 2012).

The chemical reactions of **juniper EO** from *Juniperus communis* L. (Cupressaceae) under different storage conditions was studied by Odak et al. (2018). The EO was stored for one year in daylight or dark, at room temperature or in the refrigerator (4°C), with or without oxygen availability. Samples were tested after one, two and twelve months. The most striking transformation was a decline in verbenone content under influence of light, both under nitrogen or oxygen atmosphere. Verbenone is an unsaturated bicyclic monoterpene ketone, which is photochemically reactive. Among the photoreaction products were piperitone and isopiperitone, as well as some small unidentified peaks. Furthermore, β-myrcene decreased under all applied conditions, with the alteration being less conspicuous if the sample was stored in the dark and under nitrogen, and limonene apparently oxidized to form α-terpineol, when oxygen was available, even in the dark. But after all, changes of juniper EO were neither rapid nor quantitatively impressive, therefor it can be concluded that juniper EO is mostly stable, especially under storage conditions that exclude oxygen (Odak et al., 2018).

Odak et al. (2018) also investigated **immortelle EO** sourced from *Helichrysum italicum subsp. italicum* (Roth., Asteraceae) by submitting it to the same storage conditions as

they did with juniper EO. When exposed to light, immortelle EO showed photosensibility, given that the amount of italicene and isoitalicene increased from 4.9% to 6.5% and from 0.4% to 1.0%, respectively, within 12 months, while in the dark, quantities remained unchanged. Transformations were even more distinct under storage in nitrogen atmosphere. Also, a decrease in caryophyllene has been noticed. According to these results, it seems recommendable that immortelle oil be stored in the absence of light (Odak et al., 2018).

2.5. Influence of reaction conditions

Effect of solvent

Naturally, the solvent medium, in which any photochemical reactions occur, does play a certain role. It could alter the speed or type of reactions due to interactions with the constituents of the EO solved within. Excess vibrational energy is removed by the medium, making it act like a heat sink (Michl, 1974).

Kejlová et al. (2007) assessed the phototoxicity of bergamot oil from four different suppliers, and found that the solvent used had great impact on the results obtained by the 3T3 NRU test (see below). For this assay, official guidelines recommend ethanol or DMSO as solvent (OECD, 2004). Utilization of these yielded ambiguous borderline phototoxicity values for two of the four samples, whereas the reaction in aqueous solution (PBS) gave a clear phototoxic classification. Moreover, in another study, Kejlová et al. (2010) tested the phototoxic potential of EOs of lemon, orange and *Litsea cubeba* (Pers., Lauraceae), and came to the same conclusion. DMSO poses a typical hydroxyl radical scavenger, thus it may even attenuate the phototoxic effect of tested substances. Therefore, the phototoxicity assessment without solubilizers might be advisable even for EOs with limited solubility in water, to prevent underpredictive classifications due to these radical scavenging effects (Kejlová et al., 2007).

In the RBC PT (see below), phosphate buffered saline (PBS) is being used for sample preparation, and ethanol and DMSO at 10% final test concentration are recommended as vehicles (Pape et al., 2001).

Beltrame et al. (2013) found that the solvent medium of the EO (in their case, marjoram EO) modified the pattern of decomposition by UV radiation. Ethanol and hexane proved

to be rather similar in their absorbance levels, indicating same chemical degradation mechanisms, whereas EO in dichloromethane showed slightly different behavior. In the study of Tateba et al. (1993) the photoconversion of *cis*- and *trans*-jasmone increased in ethyl acetate (aprotic solvent), compared to methanol and ethanol (protic solvents).

Effect of duration and intensity

The higher the temperature, the higher the yield, naturally (Castro et al., 2010), and according to the Van't Hoff law a temperature elevation of 10K doubles the reaction rate, as explained in Glasl (1975). But when it comes to the influence of duration and intensity of light irradiation, opinions differ and no standard procedure seems available.

Beltrame et al. (2013) exposed marjoram EO to UV radiation for only 5 minutes, using a photoreactor with a 125W mercury lamp, or a 250W lamp for accelerated photodegradation, both with removed outer bulb to allow UV light emission, and took readings every minute. Castro et al. (2010) left their samples in the photoreactor for 120 minutes, under constant stirring. In the study by Dijoux et al. (2006), the cells underwent UV-A/visible light treatment at 1.6– 1.8 mW/cm^2 for 50min. Turek/Stintzing (2012) submitted their samples to simulated daylight conditions by means of 24 hours per day irradiation with 5000 lx fluorescent tubes from Osram L36W/840, for up to 24 weeks.

The ICH (2012) stated that UV-A doses ranging from 5 to 20 J/cm^2, i.e. UV-A dosage comparable to that procured during extensive outdoor activities on days in summer around noon time in temperate zones at sea level, are effectively used in up-to-date *in vitro* and *in vivo* phototoxicity assays. In the photohaemolysis study by Placzek et al. (2007), samples were exposed to 0, 5, 25, 50 or 100 J/cm^2 UV-A (UVASUN 5000) or to 0 (0), 500 (0.2), 1000 (0.4) or 2000 (0.8) mJ/cm2 UV-B (J/cm^2 UV-A). The RBC photohaemolysis test by Pape et al. (2001) demanded an intensity of 15 J/m^2 UV-A and approximately 1 J/m^2 UV-B, since human erythrocytes can be exposed to more-intensive UV-B irradiation than other cell lines, due to their specific cellular defense. The protocol of the *in vitro* 3T3 NRU assay stipulates an exposure of 5 J/cm^2 in the UV-A range for 50 minutes for the assessment of phototoxicity (OECD, 2004), suggesting that this duration and intensity should be sufficient for phototoxic effects to be revealed.

3. Phototoxicity

For human skin, solar radiation is not only a source of energy for numerous physiological processes, but also an environmental stressor. Exposure to visible light or UV light can directly cause photodermatoses, and many photoactive chemicals can induce photosensitivity (Maibach/Honari, 2014). UV-A light is able to reach capillary blood, while UV-B only penetrates the epidermis. Hence, UV-A is more relevant for the photochemical activation of systemic drugs, whereas UV-B is of clinical relevance for topical formulations on light-exposed tissues (ICH, 2012).

According to the OECD guidelines for the testing of phototoxic potential of substances, phototoxicity is defined as "*a toxic response from a substance applied to the body which is either elicited or increased (apparent at lower close levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance*" (OECD, 2004). However, acute skin responses to photosensitizing chemicals can also be photoallergic reactions. Those two processes are distinguished in photochemistry, for phototoxic reactions induce toxic cell damage and are non-immunological, but photoallergic reactions, on the other hand, are T-cell-mediated immunological reactions. Most of the substances that elicit photoallergic responses are also phototoxic (Placzek et al., 2007).

It can occur as an adverse reaction to cosmetic products or pharmaceutical drugs, and therefore it is an utmost necessity to evaluate the phototoxic potential of the ingredients of such, so as not to put the patient or user at risk. The use of *trans*-anethole, a major constituent in some EOs, for example, has been the subject of discussion, for when irradiated with UV light, *cis*-anethole is formed, which does not only possess an unpleasant scent and flavor, but is also toxic (Castro et al., 2010).

3.1. Methods for the *in vitro* assessment of phototoxicity

To determine phototoxicity of chemicals, initially their ability of UV/visible light (290-700nm) absorption is assessed. For this, the molar extinction coefficient (MEC) is used, a constant for any given molecule under a standard set of conditions like solvent, wavelength

and temperature, which mirrors the photon absorption efficiency of the molecule (ICH, 2012). Subsequently, a chemical assay is utilized to measure reactive oxygen species (ROS), which can help predict phototoxicity, as they are generated by a photoreactive chemical upon exposure to light (Onoue, 2008). Apparently, ROS assays have low specifity, giving many false positive results, but on the other hand proving high sensitivity, hence a negative result in this assay would indicate a very low risk of phototoxicity potential (ICH, 2012).

Ultimately, there are several different methods available for the assessment of phototoxicity. All include a certain human or animal cell line, the application of the substance to be tested plus treatment with irradiation, and subsequently a test on cell viability to determine the effect. When the *in vitro* phototoxicity assay shows positive results, further testing is required, utilizing reconstituted 3D human skin models or *in vivo* preclinical trials (Maibach/Honari, 2014).

3T3 NRU assay

The 3T3 cell neutral red uptake test is a common colorimetric method of assessing phototoxic potential, and currently the only one validated by the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM, 2018a). It is known to be highly sensitive, specific and reproducible and identifies substances that either act as photoirritants after dermal application or show phototoxic effects after systemic administration (Dijoux et al., 2006). The 3T3 NRU assay is commonly used in chemical and cosmetics industries as it is regarded as reliable in its capacity to predict acute phototoxicity effects *in vivo* (OECD, 2004).

This assay uses monolayer cell cultures of mouse fibroblast cell line Balb/c 3T3 to assess the cytotoxicity of a compound with or without exposure to a non-cytotoxic dose of simulated sunlight (5 J/cm^2 in the UV-A range for 50 min). The nondiffusion uptake of neutral red (= NR, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride), a weak cationic dye, into the cells and subsequent accumulation in lysosomes is measured by a spectrophotometer. Cells damaged by the phototreatment show a decreased uptake, therefore viable, damaged and dead cells can be distinguished. To evaluate the data, the photoirritation factor (PIF, see below) and mean photoeffect (MPE, see below) are calculated

and the results of photoexposed samples is compared to the photoprotected ones (Spielmann et al., 1998; OECD, 2004).

Previous studies have found NRU test and MTT conversion test to be mainly equivalent in results, and rabbit cornea derived SIRC cells or human keratinocytes can be used instead of the murine fibroblastic 3T3 cells (Dijoux et al., 2006), therefore, those variations of the assay could probably be used as well, but the only validated method remains the one utilizing the 3T3 NRU assay.

Photohaemolysis test and RBC PT

First there was the photohaemolysis test, an early protocol, in which the haemoglobin released from cells damaged by phototoxicity was determined by converting it to its stable form by chemical oxidation and then measuring it photometrically. A disadvantage of this test was that it did not include the measurement of met-haemoglobin, the formation of which poses another important endpoint of the red blood cell treatment with phototoxic chemicals under light exposure. Then, Pape et al. (1994) improved the original photohaemolysis assay and introduced the red blood cell phototoxicity test (RBC PT). This new approach is designed for the combined testing for the ability of potentially phototoxic substances to haemolyze erythrocyte membranes and/or oxidize haemoglobin under UV irradiation (Pape et al., 2001).

In the course of this assessment, suspensions of isolated human erythrocytes are subjected to the test compounds and exposed to UV light for 150 minutes. After ensuing 30 minutes of incubation in the dark, changes in optical density are measured at 525nm and 630nm to determine the photohaemolysis and haemoglobin oxidation, respectively (Pape et al., 2001). Subsequently, the photohaemolysis factor (PHF) is calculated as the ratio of the H50-values of the samples incubated in the dark and the ones irradiated. Substances with a PHF ≥ 3 are considered phototoxic. As for haemoglobin oxidation, the met-haemoglobin formation is conveyed as maximal change in the optical density (ΔOD_{MAX}) at 630nm. If $\Delta OD_{MAX} \geq 0.05$, the substance is assumed to possess phototoxic potential (Pape et al., 2001).

The RBC PT assay passed the prevalidation process during the EU/COLIPA validation programme, which was designed to examine the suitability as regulatory test on phototoxicity (Pape et al., 2001). Erythrocytes have specific cellular defense mechanisms and are therefore not as sensitive to UV-B irradiation as other cell lines (Pape et al., 2001), which is an advantage compared to the 3T3 NRU assay. Also, the RBC PT assay grants additional information about the mechanism of phototoxicity, as compounds will yield a negative result if their cytotoxic mechanism under irradiation does not include the formation of reactive oxygen species. Therefore, this test poses a useful additional *in vitro* test method, especially for mechanistic studies (Liebsch et al., 2005).

Reconstituted 3D human skin models

Reconstituted skin models are 3D biostructures consisting of cultured normal human keratinocytes (Maibach/Honari, 2014), and possess a structure very similar to *in vivo* human epidermis. Currently, there are several 3D skin models available, such as EpiDerm™, EpiSkin™ and SkinEthic™. EpiDerm™, for example, has a basal cell layer topped on a support filter, above that spinosum cells, then finally a clear granulosum layer with a stratum corneum at the upper surface (Jones, 2008).

Similar to monolayer assays such as 3T3 NRU, the human skin models work with the premise that phototoxic chemicals will damage cells when exposed to light and can therefore be assessed by measuring the cell viability after irradiation treatment. The cell viability is here scaled by MTT conversion test (Maibach/Honari, 2014). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (**MTT**) assay is based on the uptake of the soluble yellow MTT tetrazolium salt by mitochondrial succinic dehydrogenase and its subsequent reduction to an insoluble blue MTT formazan product. This reaction is dependent on the mitochondrial function and therefore an indicator of cell viability (Dijoux et al., 2006).

Due to their high cost, they are not part of standard testing operations, but reconstituted human skin models allow for a range of topically applied substances, from single chemicals to final pharmaceutical formulations to be tested (Jones, 2008). In comparison to monolayer cell cultures, there are less solubility problems because of the direct application of chemicals to the skin surface, also, higher concentrations can be tested, and the 3D structure

of the model allows for higher proportion of UV-B, thus rendering a more accurate solar irradiation model possible (Jones, 2001; Jones, 2008). These assays can measure cell viability with or without phototreatment. They might be close to reality, but they can still be less sensitive than human skin *in vivo* (ICH, 2012). EpiDerm^{TM} reconstituted human skin models showed promising results in a prevalidation study (EURL ECVAM, 2018a) and are recommended as further investigations to determine actual hazard when phototoxic effects are solely observed at the highest test concentration in the 3T3 NRU test (OECD, 2004). Also, they are validated as methods for the assessment of skin irritation, i.e. a local inflammatory reaction of the skin caused by the non-specific immune system after application of an irritant (EURL ECVAM, 2018b).

Photo-irritation factor (PIF) and mean photo effect (MPE)

PIF and MPE are values used to assess the phototoxic potential of chemical substances. The photo-irritation factor (PIF) is defined as the difference between IC50 in presence and absence of UV light (Dijoux et al., 2006). The mean photo effect (MPE) is predicated on the comparison of the complete concentration response curves. Concerning the assessment of the results, a PIF<2 or an MPE<0.1 predicts "no phototoxicity". A PIF>2 and <5 or an MPE >0.1 and <0.15 suggests "probable phototoxicity" (Spielmann et al., 1998; OECD, 2004), although according to an ICH guideline on the photosafety evaluation of pharmaceuticals "compounds in this category generally do not warrant further photosafety evaluations" (ICH, 2013). In the case of PIF>5 or an MPE>0.15 "phototoxicity" is forecasted (Spielmann et al., 1998; OECD, 2004).

In a study comparing different systems for the assessment of phototoxic potential, Dijoux et al. (2006) came to the conclusion, that NRU assay and MTT conversion test on both rabbit-cornea derived SIRC cells and murine fibroblastic 3T3 cells were all able to differentiate between phototoxic (PIF>5) and non-phototoxic (PIF<5) molecules/oils, but had problems distinguishing mildly or probably phototoxic substances with 2<PIF<5 from non-phototoxic ones with PIF<2 correctly (Dijoux et al., 2006).

3.2. Phototoxic essential oils and essential oil constituents

Most phototoxic EOs are found in the botanical families of Apiaceae and Rutaceae, probably due to evolutionary divergence. Apart from those, phototoxic EOs may be found prevalently in the Asteraceae and Moraceae families (Tisserand/Young, 2014).

As aforementioned, substances can be phototoxic and/or provoke T-cell-mediated photoallergic reactions, both of which resulting in skin irritation similar to that of an acute sunburn. Within minutes to hours of sun exposure, agonizing erythema may develop on irradiated skin (Maibach/Honari, 2014). Most phototoxic compounds absorb energy from UV-A radiation leading to the generation of activated derivatives capable of inducing cellular damage (Dijoux et al., 2006). To afflict cutaneous inflammation, chemicals need to be capable of transgression into the epidermis and there must bind to proteins. The allergenic potential of EOs can be accredited to hydroperoxide derivatives of terpenoids, which are generated by (photo-)oxidation (Turek/Stintzing, 2013). The non-oxidized originals, on the other hand, were found to be not or only slightly irritating (Pirilä/Siltanen, 1958; Hausen et al., 1999; Matura et al., 2005; Karlberg et al., 2008; Bråred-Christensson et al., 2009).

A class of substances often brought into connection with phototoxicity of EOs are the furocoumarins. They are synthesized and used by plants as defensive chemicals and are characterized by their coumarin structure conjoined with a furan ring. Depending on its position, they can be differentiated into two subtypes, the linear psoralen-type and the angular angelicin-type (see Fig.16). Furocoumarins like psoralen, bergapten (= 5-MOP), xanthotoxin and angelicin, which are abundant in the Apiaceae, Rutaceae (for example some *Citrus* species), Moraceae and other families, are known to be phototoxic and also carcinogenic under UV irradiation. (Fu et al., 2013). Others, like bergamottin, bergaptol, isobergapten and isopimpinellin are non-phototoxic. Furocoumarins are larger than most EO constituents, but can pass over during steam-distillation anyway. Still, cold-pressed EOs show much higher content of these compounds than steam-distilled ones. Commonly available EOs that are known to be phototoxic include the EOs of angelica root, bergamot (cold pressed), bitter orange (cold pressed), cumin, fig leaf absolut, grapefruit (cold pressed), lemon (cold pressed), lime (cold pressed), mandarin leaf, opopanax, rue and

tagetes. The following might be phototoxic: clementine (cold pressed), combava fruit, skimmia, angelica root, celery leaf and seed, cumin seed, khella, lovage leaf and parsnip (Tisserand/Young, 2014).

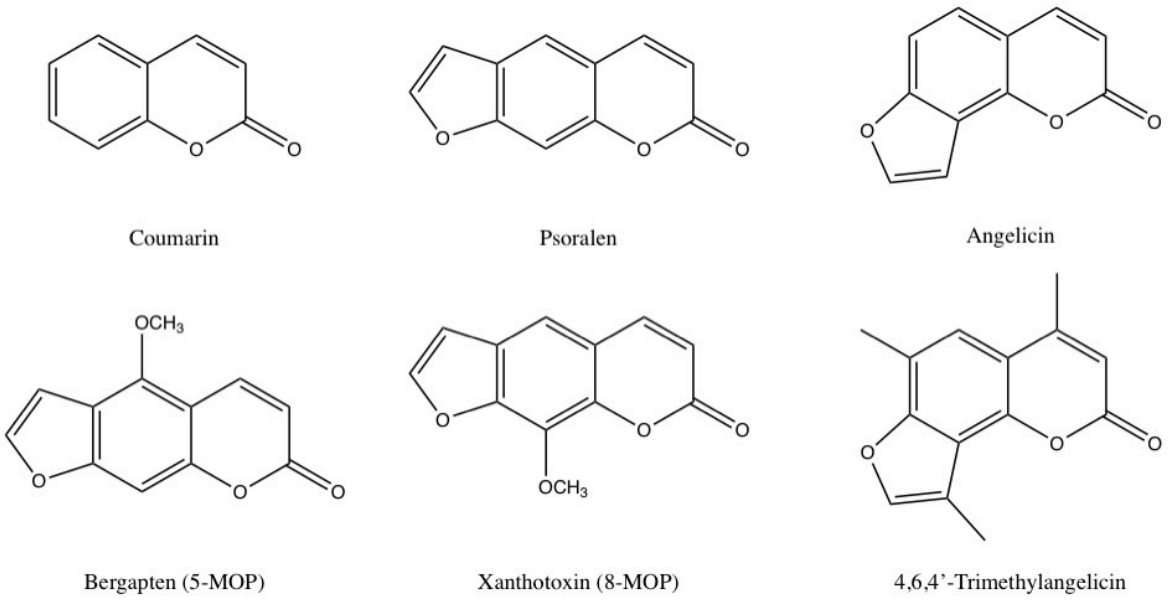

Figure 16: Some selected furocoumarins (adapted and newly drawn from Fu et al., 2013; Kinley et al., 1994)

Three distinct steps of reactions occur between psoralens and DNA: Firstly, the non-covalent intercalation of the psoralen between DNA base pairs, secondly, the photochemical reaction of psoralen with a pyrimidine base, resulting in a monoadduct, and lastly, an interstrand cross-link formed by absorption of another photon. The interstrand cross-links in particular are thought to be responsible for photosensitizing effects. For example, 6,4,4′-trimethylangelicin (TMA, see Fig.16), a well-known photosensitizing agent, was found to induce interstrand cross-links in mammalian cell DNA (Miolo et al., 1989; Bordin et al., 1994).

The photocycloaddition of a psoralen (8-MOP or 4,5',8-trimethylpsoralen) to a pyrimidine yields a cyclobutane ring with four asymmetric centers, whereas the stereochemistry is determined by geometrical limitations inflicted by the DNA helix during the formation of the intercalation complex before UV irradiation and cycloaddition. Therefore, all effectively formed adducts have *cis-syn* stereochemistry. But the most abundant products are two diastereomeric thymidine adducts, which are generated by

photocycloaddition between the psoralen´s 4',5' (furan) double-bond and the 5,6 double-bond of the pyrimidine (Kanne et al., 1982).

8-Methoxypsoralen (= 8-MOP, methoxsalen) is infamous for its phototoxic effects, which is put to a therapeutic use in photochemotherapy and PUVA (psoralen + UV-A) therapy for patients suffering from psoriasis and other skin diseases. In the process of this therapy, UV-A light energy is absorbed by 8-MOP, and the excited photosensitizer in the triplet state passes energy to molecular oxygen, forming singlet oxygen (type II mechanism), or transfers an electron, which results in the generation of a superoxide anion radical (type I mechanism) (Ochsner, 1997). Either way, reactive oxygen species (ROS) are created, increasing the oxidative stress in the cell, which leads to the activation of the complement system, the induction of apoptosis or necrosis, and subsequently to cell death. Ironically, although 8-MOP and psoralen are used to treat skin illnesses, they can also cause such: PUVA therapy has been found to increase the risk of human skin tumors over the years (Stern et al., 1997; Stern, 2001; Katz et al., 2002).

Also, Young et al. (1990) studied the phototumorigenicity of 5-methoxypsoralen (= 5-MOP, bergapten), a constituent of bergamot (*Citrus bergamia* Risso/Poit.) oil, by means of model perfumes containing this oil. They concluded that 5-MOP indeed has phototumorigenic potential, already at about 5ppm. Sunscreens were able to significantly lower the tumorigenicity (Young et al., 1990).

The chemical profile and photoinduced cytotoxicity of the EO of *Citrus medica* L. cv. Diamante peel was studied by Menichini et al. (2010). The most abundant compounds were found to be limonene, γ-terpinene, citral, geranial, β-pinene and α-pinene. The oil also comprised two coumarins, bergapten and citropten. After 100min of exposure to UV light, the EO showed cytotoxic activity. The phototoxic effect was mainly ascribed to bergapten, as the strong antiproliferative effect of bergapten was not found with citropten (Menichini et al., 2010).

Dijoux et al. (2006) determined in a 3T3 NRU phototoxicity assay that orange EO was "probably" phototoxic, and cytotoxic in the absence of UV light. A study by Binder et al. (2016) supports this result, as they came to the conclusion that orange oil was phototoxic even at low concentrations. Lemongrass EO showed slight phototoxic and cytotoxic prop-

erties, whereas sandalwood EO was strongly cytotoxic without UV radiation, but not phototoxic. Carrot and ginger EOs were also not phototoxic, but faintly cytotoxic. To which components the orange and lemongrass EOs owe their phototoxicity remains to be investigated (Dijoux et al., 2006).

The EO of *Anthemis nobilis* L. (or nowadays *Chamaemelum nobile L.*, Asteraceae) caused barely perceptible erythema after irradiation in a study of Forbes et al. (1977), where they used hairless mice and miniature swine for testing, and was thus classified as non-phototoxic. 8-Methoxypsoralen, on the other hand, was used as a control and found to be phototoxic (Forbes et al. quoted in Johnson et al., 2017).

Gallucci et al. (2010) investigated *Eugenia uniflora* L. (Myrtaceae) leaf EO using the 3T3 NRU test and results showed a PIF<2. They concluded that its use as a fragrance ingredient raises no safety concerns.

A safety assessment study highlighted that undiluted *Mentha piperita* L. (Lamiaceae) oil does evoke moderate to severe reactions in rabbits after repeated intradermal application, but does not appear to be phototoxic. After thoroughly investigating several studies, the CIR expert panel concluded that peppermint oil is safe as used in cosmetic formulations, but cautions to keep the concentration of the constituent pulegone under 1%, because of its toxicity (CIR expert panel, 2001).

Conclusion

Comprising a range of lipophilic and volatile constituents derived from many different chemical classes, EOs are known to be susceptible to conversion and degradation reactions (Turek/Stintzing, 2013). The stability of EOs depends on several internal factors like chemical structure and impurities, as well as external factors like temperature, exposure to humidity, oxygen, or light (Khayyat/Roselin, 2018).

When studying the degradation of EOs, metallic catalysts, molecular oxygen or photosensitizers are often used to accelerate the occurrence of oxidation reactions, therefore the transformations discovered in such a simulation may not necessarily mirror realistic circumstances. Furthermore, it must be noted that single compounds may not react in the same way as complex mixtures such as EOs, because the different constituents can affect the behavior of the others. On account of this, the findings may not be directly transferable, rendering utilization of single compounds as surrogates or references for EOs questionable (Turek/Stintzing, 2013).

Depending strongly on the exact photoreaction circumstances, photosensitive EOs may be altered in their qualitative and quantitative composition. For instance, if oxygen is available to the reaction mixture, photoxidations are more likely to take place, as with geraniol (Mori/Iwahashi, 2016) or citral (Li et al., 2016). Meanwhile, in the case of hydrogen peroxide presence, photoepoxidation has a higher prevalence, for instance in the photoreactions of pulegone and camphene (Elgendy/Khayyat, 2008). When the irradiated mixture is being deprived of oxygen, there are still other reaction pathways possible, including isomerizations, polymerizations and cycloadditions. Generally, there is a noticeable influence of the solvent used. DMSO, for example, acts as a hydroxyl radical scavenger, and thus may be able to quench the phototoxic effect of tested substances, as photoreaction progress is inhibited (Kejlová et al., 2007).

Photoinitiation of chemical reactions can be used in positive applications, as for specific elimination by photodegradation of unwanted molecules in complex mixtures such as EOs (Moulin et al., 1995), or as a tool for the drug design of anticancer agents and potent chemoprevention, for example the photoepoxidation of safranal (Khayyat/Elgendy, 2018). But when transformations are triggered unknowingly, in a product where they should not,

it can be a nuisance, or in the worst case, even potentially dangerous for the consumer. Transformations of EOs and their constituents due to UV light can have different consequences: something as basic as a change in viscosity, odor and flavor, like in the example of pummelo EO (Li et al., 2016), but also the generation of toxic photoproducts that can harm the consumer, as with the isomerization of *trans*-anethole to toxic *cis*-anethole (Castro et al., 2010) and the phototumorigenic potential of bergapten (= 5-MOP) from bergamot oil (Young et al., 1990). Interestingly, some biological activities of EOs can be enhanced by irradiation with light: the photodimerization products of cinnamaldehyde, safrole and eugenol elicit stronger inhibiting effects against *Candida albicans* than their corresponding monomers (Khayyat, 2013), and the antibacterial activity of the monoepoxy and diepoxy derivatives of safranal and the hydroperoxides of some monoterpenes (α-pinene, β-pinene and limonene) are higher than those of their pre-phototransformation parent molecules (Khayyat, 2013; Chalchat et al., 2000; Marqués-Calvo et al., 2017).

In general, there is no risk of phototoxicity to be expected if the EO is used in products which are either not applied to the body, or washed off the skin directly after application, such as soaps or shampoos. Moreover, when the EO product is applied to skin that is covered in a way to prevent UV rays from reaching it, there is also no risk of phototoxic reactions. After usage of potentially phototoxic EO products, it is advisable to protect the respective skin areal from UV radiation for at least 12 to 18 hours (Tisserand/Young, 2014).

As for the prevention of EO degradation during storage, they should be stored in tightly closed, dark glass vials in a cool place to ensure lasting quality (Buckle, 2003; Clarke, 2008). The headspace should be reduced to a minimum, to avoid contact with oxygen and therefore oxidation reactions, and there should be no water residues (Kaul, 1997). Metal contaminants, especially heavy metal copper and ferrous ions, should be avoided, as they are considered to catalyze autoxidation and the formation of singlet oxygen (Choe/Min, 2006).

Strategies of EO shielding are being developed, which could improve shelf-life of EO products: the nanoemulsion technique, where the EO droplets are encased in propolis (Gismondi, 2014) or gum arabic (Bertolini, 2001), for instance, or cyclodextrin encapsulation, where inclusion complexes are formed in order to minimize unwanted transformations as well as losses due to evaporation, and increase chemical stability (Marques, 2010).

Table of Figures

References / Bibliography

Bakkali F., S. Averbeck, D. Averbeck, M. Idaomar. (**2008**) Biological effects of essential oils - a review. *Food Chem Toxicol*, 46/2, 446-475. DOI:10.1016/j.fct.2007.09.106.

Beltrame J.M., R.A. Angnes, L.U. Rovigatti Chiavelli et al. (**2013**) Photodegradation of essential oil from Marjoram (Origanum majorana L.) studied by GC-MS and UV-VIS spectroscopy. *Rev. Latinoamer. Quím*., 41/2, 81-88.

Bertolini A.C., A. C. Siani, C. R. Grosso. (**2001**) Stability of monoterpenes encapsulated in gum Arabic by spray-drying. *J Agric Food Chem*, 49/2, 780–785.

Bråred-Christensson J., P. Forsström, A.-M. Wennberg et al. (**2009**) Air oxidation increases skin irritation from fragrance terpenes. *Contact Dermatitis*, 60/1, 32–40.

Binder S., A. Hanáková, K. Tománková et al. (**2016**) Adverse phototoxic effect of essential plant oils on NIH 3T3 cell line after UV light exposure. *Cent Eur J Public Health*, 24/3, 234-240. DOI:10.21101/cejph.a4354.

Bordin F., C. Marzano, C. Gatto et al. (**1994**) 4,6,4′-Trimethylangelicin induces interstrand cross-links in mammalian cell DNA. *Journal of Photochemistry and Photobiology B: Biology*, 26/ 2, 197-201. https://doi.org/10.1016/1011-1344(94)07040-7.

Buchbauer G. and M. Erkic. (**2015**) *Antioxidative Properties of Essential Oils and Single Fragrance Compounds*. in K.H.C. Başer and G. Buchbauer: "Handbook of essential oils: science, technology and applications", Second Edition, CRC Press.

Buckle J. (**2003**) *Clinical aromatherapy: essential oils in practice*, Second Edition, Churchill Livingstone: Edinburgh.

Castro H.T., J.R. Martínez, E. Stashenko. (**2010**) Anethole isomerization and dimerization induced by acid sites or UV irradiation. *Molecules*, 15, 5012-5030. DOI:10.3390/molecules15075012

Chalchat JC, Chiron F, Garry RP, J. Lacoste J, Sautou V. (**2000**) Photochemical hydroperoxidation of terpenes. Antimicrobial activity of α-pinene, β-pinene and limonene hydroperoxides, *Journal of Essential Oil Research*, 12/1, 125-134, DOI:10.1080/10412905.2000.9712059

Chellappandian M., P. Vasantha-Srinivasan, S. Senthil-Nathan et al. (**2018**) Botanical essential oils and uses as mosquitocides and repellents against dengue. *Environment International*, 113, 214-230. DOI:10.1016/j.envint.2017.12.038

Chiang H. and S. Li. (**1978**), Studies on the photodimerization of isoeugenol. *Jnl Chinese Chemical Soc*, 25, 141-147. DOI:10.1002/jccs.197800024

CIR Cosmetic Ingredient Review Expert Panel (**2001**) Final report on the safety assessment of Mentha piperita (peppermint) oil, Mentha piperita (peppermint) leaf extract, Mentha piperita (peppermint) leaf, and Mentha piperita (peppermint) leaf water. *International Journal of Toxicology*, 20/Suppl.3, 61–73.

Clarke S. (**2008**) *Chapter 8 – Handling, safety and practical applications for use of essential oils*. in S. Clarke: "Essential chemistry for aromatherapy", Second Edition, Churchill Livingstone Elsevier, eBook. https://doi.org/10.1016/B978-0-443-10403-9.00008-X

Choe E. and D.B. Min. (**2006**) Mechanisms and factors for edible oil oxidation. *Comprehensive Reviews in Food Science and Food Safety*, 5, 169-186. DOI:10.1111/j.1541-4337.2006.00009.x

Dhifi W., S. Bellili, S. Jazi et al. (**2016**) Essential oils' chemical characterization and investigation of some biological activities: a critical review. *Medicines (Basel)*, 3/4, 25. DOI:10.3390/medicines3040025

Dijoux N., Y. Guingand, C. Bourgeois et al. (**2006**) Assessment of the phototoxic hazard of some essential oils using modified 3T3 neutral red uptake assay. *Toxicology in Vitro,* 20, 480–489.

Elgendy E.M. and S.A. Khayyat. (**2008**) Oxidation studies on some natural monoterpenes: citral, pulegone, and camphene. *Russ J Org Chem,* 44/6, 814-822. https://doi.org/10.1134/S1070428008060067

European Monitoring Centre for Drugs and Drug Addiction EMCDDA (**2016**): EU drug market reports, Chapter 6: Amphetamine, methamphetamine and MDMA — Production and precursors http://www.emcdda.europa.eu/publications/eu-drug-markets/2016/online/amphetamines-ecstasy/production-precursors_en [accessed Dec 30th 2018]

European Union Reference Laboratory for alternatives to animal testing EURL ECVAM (**2018a**) Alternative methods for toxicity testing – Validated test methods – Phototoxicity https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/validated-test-methods/phototoxicity [accessed November 4th 2018]

European Union Reference Laboratory for alternatives to animal testing EURL ECVAM (**2018b**) Alternative methods for toxicity testing – Validated test methods – Skin irritation https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/validated-test-methods/skin-irritation [accessed November 4th 2018]

Figueiredo A.C., J.G. Barroso, L.G. Pedro, J.J. Scheffer. (**2008**) Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour Fragr. J.*, 23, 213-226. DOI:10.1002/ffj.1875

Forbes P.D., F. Urbach, R.E. Davies. (**1977**) Phototoxicity testing of fragrance raw materials. *Food and Cosmetics Toxicology*, 15/1, 55-60. https://doi.org/10.1016/S0015-6264(77)80264-2.

Fu P.P., Q. Xia, Y. Zhao et al. (**2013**) Phototoxicity of herbal plants and herbal products. *Journal of Environmental Science and Health*, Part C, 31/3, 213-255. DOI:10.1080/10590501.2013.824206

Gallucci S., A. Placeres Neto, C. Porto et al. (**2010**) Essential oil of Eugenia uniflora L.: an industrial perfumery approach. *Journal of Essential Oil Research*, 22/2, 176-179. DOI:10.1080/10412905.2010.9700296

Gismondi A., L. Canuti, M. Grispo, A. Canini. (**2014**) Biochemical composition and antioxidant properties of lavandula angustifolia Miller essential oil are shielded by propolis against UV radiations. *Photochemistry and Photobiology*, 90, 702–708.

Glasl H. (**1975**) Über die Haltbarkeit von Terpenoiden in Extrakten und Lösungen mit unterschiedlichem Alkoholgehalt. *Archiv der Pharmazie*, 308/2, 88-93.

Habtemariam S. (**2018**) Antidiabetic potential of monoterpenes: a case of small molecules punching above their weight. *International Journal of Molecular Sciences,* 19/1, 4. DOI:10.3390/ijms19010004

Hänsel R. and O. Sticher. (**2010**), *Pharmakognosie Phytopharmazie*, 9. Aufl., Springer: Heidelberg.

Hausen B.M., J. Reichling, M. Harkenthal. (**1999**) Degradation products of monoterpenes are the sensitizing agents in tea tree oil. *Am J Cont Derm*, 10/2, 68–77.

Heldt H.W. and B. Piechulla. (**2011**) *Chapter 16 - Secondary metabolites fulfill specific ecological functions in plants*. in H.W. Heldt and B. Piechulla: "Plant Biochemistry", Fourth Edition, Academic Press, 399-408. DOI:https://doi.org/10.1016/B978-0-12-384986-1.00016-8.

ICH International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (**2012**) Guidance on photosafety evaluation of pharmaceuticals S10 Step 2 version 13. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM337572.pdf.

ICH International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (**2013**) Harmonised tripartite guideline "Photosafety evaluation of pharmaceuticals S10" http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S10/S10_Step_4.pdf [accessed March 31st 2018]

Irmak S., K. Solakyildirim, A. Hasanoğlu, O. Erbatur. (**2010**) Study on the stability of supercritical fluid extracted rosemary (*Rosmarinus Offcinalis* L.) essential oil. *Journal of Analytical Chemistry*, 65: 899-906. DOI:10.1134/S1061934810090030}

IUPAC International Union of Pure and Applied Chemistry (**1997**) Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications:Oxford. XML online corrected version: http://goldbook.iupac.org (2006-) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. https://doi.org/10.1351/goldbook

Ivanitskikh A.S. and I.G. Tarakanov. (**2014**) Effect of light spectral quality on essential oil components in Ocimum basilicum and Salvia officinalis plants. *International Journal of Secondary Metabolite*, 1/1, 19.

Iwanami Y., H. Tateba, N. Kodama, K. Kishino. (**1997**) Changes of lemon flavor components in an aqueous solution during UV Irradiation. *Journal of Agricultural and Food Chemistry*, 45, 463−466.

Johnson W., B. Heldreth, W.F. Bergfeld et al. (**2017**) Safety assessment of Anthemis nobilis-derived ingredients as used in cosmetics. *International Journal of Toxicology*, 36/Suppl.1, 57-66. DOI:10.1177/1091581817705620

Jones P., W.W. Lovell, A.V. King, L.K. Earl. (**2001**) In vitro testing for phototoxic potential using the EpiDerm™ 3-D reconstructed human skin model. *Toxicology Methods*, 11, 1–19. DOI:10.1080/105172301300055115

Jones P. (**2008**) *In vitro Phototoxicity Assays*. in R.P. Chilcott and S. Price: "Principles and Practice of Skin Toxicology". DOI:10.1002/9780470773093.ch10

Kanne D., K. Straub, H. Rapoport, J.E. Hearst. (**1982)** The psoralen-DNA photoreaction. Characterization of the monoaddition products from 8-methoxypsoralen and 4,5',8-trimethylpsoralen. *Biochemistry*, 21/5, 861-871. DOI:10.1021/bi00534a008

Karlberg A.-T., M.A. Bergström, A. Börje et al. (**2008**) Allergic contact dermatitis – formation, structural requirements, and reactivity of skin sensitizers. *Chem Res Toxicol*, 21/1, 53–69.

Katz K.A., I. Marcil, R.S. Stern. (**2002**) Incidence and risk factors associated with a second squamous cell carcinoma or basal cell carcinoma in psoralen + ultraviolet A light-treated psoriasis patients. *J Invest Dermatol.*, 118, 1038–1043.

Kaul P.N., B.R. Rajeswara Rao, A.K. Bhattacharya et al. (**1997**) Changes in chemical composition of rose-scented geranium (Pelargonium sp.) oil during storage. *Journal of Essential Oil Research*, 9/1, 115-117. DOI: 10.1080/10412905.1997.9700729

Kejlová K., D. Jírová, H. Bendová et al. (**2007**) Phototoxicity of bergamot oil assessed by in vitro techniques in combination with human patch tests. *Toxicol In Vitro*, 21/7, 1298–1303. DOI:10.1016/j.tiv.2007.05.016

Kejlová K., D. Jírová, H. Bendová et al. (**2010**) Phototoxicity of essential oils intended for cosmetic use. *Toxicology in Vitro*, 24/8, 2084-2089. https://doi.org/10.1016/j.tiv.2010.07.025.

Khayyat S.A. (**2013**) Photosynthesis of dimeric cinnamaldehyde, eugenol, and safrole as antimicrobial agents. *Journal of Saudi Chemical Society*, 17/1, 61-65. DOI:https://doi.org/10.1016/j.jscs.2011.07.014.

Khayyat S.A. (**2018**) Thermal, photo-oxidation and antimicrobial studies of linalyl acetate as a major ingredient of lavender essential oil. *Arabian Journal of Chemistry*, Article in Press. DOI:10.1016/j.arabjc.2017.12.008.

Khayyat S.A. and E. Elgendy. (**2018**) Safranal epoxide - A potential source for diverse therapeutic applications. *Saudi Pharm J*, 26/1, 115-119. DOI:10.1016/j.jsps.2017.10.004

Khayyat S.A. and L.S. Roselin. (**2018**) Recent progress in photochemical reaction on main components of some essential oils. *Journal of Saudi Chemical Society*, 22/7, 855-875. https://doi.org/10.1016/j.jscs.2018.01.008.

Kinley J.S., J. Moan, F. Dall'Aqua, A. Young. (**1994**) Quantitative assessment of epidermal melanogenesis in C3H/Tif hr/hr mice treated with topical furocoumarins and UVA radiation. *The Journal of Investigative Dermatology*, 103/1, 97-103. https://core.ac.uk/download/pdf/81943200.pdf [accessed January 10th 2019]

Krupa J., A. Olbert-Majkut, I. Reva et al. (**2012**) Ultraviolet-tunable laser induced phototransformations of matrix isolated isoeugenol and eugenol. *J. Phys. Chem. B*, 116, 11148−11158. DOI:10.1021/jp306339g

Kumari R., S.B. Agrawal, S. Singh, N.K. Dubey. (**2009**) Supplemental ultraviolet-B induced changes in essential oil composition and total phenolics of Acorus calamus L. (sweet flag). *Ecotoxicol Environ Saf*., 72/7, 2013–2019. DOI:10.1016/j.ecoenv.2009.02.006

Lewis F.D. and M. Kojima. (**1988**) Electron transfer induced photoisomerization, dimerization, and oxygenation of trans- and cis-anethole - The role of monomer and dimer cation radicals. *Journal of the American Chemical Society*, 110, 8664-8670.

Li L.J., P. Hong, F. Chen et al. (**2016**) Characterization of the aldehydes and their transformations induced by UV irradiation and air exposure of white guanxi honey pummelo (Citrus Grandis (L.) Osbeck) essential oil. *Journal of Agricultural and Food Chemistry*, 64/24, 5000-5010. DOI:10.1021/acs.jafc.6b01369

Liebsch M., H. Spielmann, W. Pape et al. (**2005**) UV-induced effects. *Alternatives to laboratory animals: ATLA*, 33/Suppl.1, 131-46.

Lis-Balchin M. and S. Hart. (**1999**) Studies on the mode of action of the essential oil of lavender (Lavandula angustifolia P. Miller). *Phytother Res*, 13/6, 540–542.

Maffei M., D. Canova, C.M. Bertea, S. Scannerini. (**1999**) UVA effects on photomorphogenesis and essential oil composition in Mentha piperita. *Journal of Photochemistry and Photobiology B-biology*, 52, 105-110.

Maibach H. and G. Honari. (**2014**) *Chapter 3 - Photoirritation (phototoxicity): clinical aspects*. in H. Maibach and G. Honari: "Applied Dermatotoxicology", 41-56. https://doi.org/10.1016/B978-0-12-420130-9.00003-7

Marques H.M. (**2010**) A review on cyclodextrin encapsulation of essential oils and volatiles. *Flavour Fragr J*, 25, 313-326. DOI:10.1002/ffj.2019

Marqués-Calvo M.S., F. Codony, G. Agustí, C. Lahera. (**2017**) Visible light enhances the antimicrobial effect of some essential oils. *Photodiagnosis and Photodynamic Therapy*, 17, 180-184. https://doi.org/10.1016/j.pdpdt.2016.12.002.

Matura M, M. Sköld, A. Börje et al. (**2005**) Selected oxidized fragrance terpenes are common contact allergens. *Contact Dermatitis*, 52/6, 320–8.

Menichini F., R. Tundis, M.R. Loizzo et al. (**2010**) In vitro photo-induced cytotoxic activity of Citrus bergamia and C. medica L. cv. Diamante peel essential oils and identified active coumarins. *Pharm Biol*, 48/9, 1059–1065. DOI:10.3109/13880200903486636

Michl J. (**1974**) *Physical basis of qualitative MO arguments in organic biochemistry*. in A. Davison, M.J.S. Dewar, K. Hafner et al.: "Topics in current chemistry 46: Photochemistry", Springer: Berlin Heidelberg.

Miguel M.G. (**2010**) Antioxidant and anti-inflammatory activities of essential oils: a short review. *Molecules*, 15, 9252-9287. DOI:10.3390/molecules15129252

Miolo G., M. Stefanidis, R.M. Santella et al. (**1989**) 6,4,4′-Trimethylangelicin photoadduct formation in DNA: production and characterization of a specific monoclonal antibody. *Journal of Photochemistry and Photobiology B: Biology*, 3/1, 101-112. https://doi.org/10.1016/1011-1344(89)80024-7

Mori H.-M. and H. Iwahashi. (**2016**) Characterization of radicals arising from oxidation of commercially-important essential oils, *Free Radical Research*, 50/6, 638-644. DOI:10.3109/10715762.2016.1162299

Morita T., K. Jinno, H. Kawagishi et al. (**2003**) Hepatoprotective effect of myristicin from nutmeg (Myristica fragrans) on lipopolysaccharide/d-galactosamine-induced liver injury. *J Agric Food Chem*, 51/6, 1560–1565. DOI:10.1021/jf020946n

Moulin C., A. Petit, J.C. Baccou. (**1995**) Selective laser photolysis of organic molecules in complex matrices. *Journal of Photochemistry and Photobioiogy A: Chemistry*, 85, 165-172.

Ochsner M. (**1997**) Photophysical and photobiological processes in the photodynamic therapy of tumours. *Journal of Photochemistry and Photobiology B: Biology*, 39/1, 1-18. https://doi.org/10.1016/S1011-1344(96)07428-3.

Odak I., T. Lukic, S. Talic. (**2018**) Impact of storage conditions on alteration of juniper and immortelle essential oils. *Journal of Essential Oil Bearing Plants*, 21/3, 614-622. DOI:10.1080/0972060X.2018.1489309

OECD Organisation for Economic Co-operation and Development (**2004**) Guidelines for the testing of chemicals, Section 4: Test no. 432: in vitro 3T3 NRU phototoxicity test. DOI:10.1787/9789264071162-en.

Onoue S., K. Kawamura, N. Igarashi et al. (**2008**) Reactive oxygen species assay-based risk assessment of drug-induced phototoxicity: classification criteria and application to drug candidates. *J Pharm Biomed Anal*, 47/4-5, 967–972. DOI:10.1016/j.jpba.2008.03.026

Pape W.J.W., M. Brandt, U. Pfannenbecker. (**1994**). Combined in vitro assay for photohaemolysis and haemoglobin oxidation as part of a phototoxicity test system assessed with various phototoxic substances. *Toxicology in Vitro*, 8, 755–757.

Pape W.J.W., T. Maurer, U. Pfannenbecker, W. Steiling. (**2001**). The red blood cell phototoxicity test (photohaemolysis and haemoglobin oxidation). EU/COLIPA Validation programme on phototoxicity (Phase II). *ATLA*, 29, 145–162.

Pelzl B., R. Wolf, B.L. Kaul. (**2018**) *Plastics, additives.* in "Ullmann's encyclopedia of industrial chemistry". DOI: 10.1002/14356007.a20_459.pub2

Pérez-Recalde M., I.E. Ruiz Ariasa, É.B. Hermida. (**2018**) Could essential oils enhance biopolymers performance for wound healing? A systematic review. *Phytomedicine*, 38, 57–65. DOI:10.1016/j.phymed.2017.09.024

Pirilä V. and E. Siltanen. (**1958**) On the chemical nature of the eczematogenic agent in oil of turpentine. III. *Dermatologica*, 117/1, 1–8. DOI:10.1159/000255561

Placzek M., W. Frömel, B. Eberlein et al. (**2007**) Evaluation of phototoxic properties of fragrances. *Acta Derm Venereol*, 87, 312–316. DOI:10.2340/00015555-0251

Prins C.L., I.J.C. Vieira, S.P. Freitas. (**2010**) Growth regulators and essential oil production. *Brazilian Journal of Plant Physiology*, 22/2, 91-102. DOI:https://dx.doi.org/10.1590/S1677-04202010000200003

Raghavan S. (**2006**) *Handbook of spices, seasonings, and flavorings*, Second Edition, CRC Press: Boca Raton.

Raut J.S. and S.M. Karuppayil. (**2014**) A status review on the medicinal properties of essential oils. *Industrial Crops and Products*, 62, 250-264. https://doi.org/10.1016/j.indcrop.2014.05.055.

Rezaee R. and H. Hosseinzadeh. (**2013**) Safranal: from an aromatic natural product to a rewarding pharmacological agent. *Iran J Basic Med Sci*, 16/1, 12-26.

Rocha S.F.R. and L. Chau Ming. (**1999**) *Piper hispidinervum: a sustainable source of safrole*. in J. Janick: "Perspectives on new crops and new uses", ASHS Press: Alexandria, VA, 479–481.
https://hort.purdue.edu/newcrop/proceedings1999/v4-479.html [accessed January 2nd 2019]

Schieberle P. and W. Grosch. (**1989**) Potent odorants resulting from the peroxidation of lemon oil. *Z Lebensm Unters Forsch*, 189/1, 26-31. https://doi.org/10.1007/BF01120443

Simmler W. (**2012**) *Photochemical Degradation*. in "Ullmann´s encyclopedia of industrial chemistry", Vol.2, Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 127-145. DOI: 10.1002/14356007.o02_o06

Spielmann H., M. Balls, J. Dupuis et al. (**1998**) The international EU/COLIPA in vitro phototoxicity validation study: results of phase II (blind trial). Part 1: the 3T3 NRU phototoxicity test. *Toxicol In Vitro*, 12/3, 305–327.

Stern R.S,. K.T. Nichols, L.H. Väkevä. (**1997**) Malignant melanoma in patients treated for psoriasis with methoxsalen (psoralen) and ultraviolet A radiation (PUVA). *New Engl J Med*, 336, 1041–1045. DOI:10.1056/NEJM199704103361501

Stern R.S. (**2001**) The risk of melanoma in association with long-term exposure to PUVA. *J Am Acad Dermatol*, 44, 755–761.

Sun H., H. Ni, Y. Yang et al. (**2014**) Sensory evaluation and gas chromatography−mass spectrometry (GC-MS) analysis of the volatile extracts of pummelo (Citrus maxima) peel. *Flavour Fragrance J.*, 29, 305−312.

Tateba H., K. Morita, W. Kameda, M. Tada. (**1993**) Photochemical reaction of (Z)-jasmone under various conditions. *Bioscience, Biotechnology, and Biochemistry*, 57/2, 220-226. DOI:10.1271/bbb.57.220

Tisserand R. and R. Young. (**2014**) *Chapter 5 - The skin.* in R. Tisserand and R. Young: "Essential Oil Safety", Second Edition, Churchill Livingstone, 69-98. DOI:10.1016/B978-0-443-06241-4.00005-9

Toda H., S. Mihara, K. Umano, T. Shibamoto. (**1983**) Photochemical studies on jasmin oil. *J Agric. Food Chem.*, 31, 554-557.

Turek C. and F.C. Stintzing. (**2012**) Impact of different storage conditions on the quality of selected essential oils. *Food Research International*, 46/1, 341-353. https://doi.org/10.1016/j.foodres.2011.12.028.

Turek C. and F.C. Stintzing. (**2013**) Stability of essential oils: a review. *Comprehensive reviews in food science and food safety*, 12, 40-53. DOI:10.1111/1541-4337.12006

Van den Bergh H. (**1986**) Light and porphyrins in cancer therapy. *Chem Br*, 22, 430-439.

World Health Organization WHO (**2018**) Health topics - Ultraviolet radiation - What is UV radiation? https://www.who.int/uv/uv_and_health/en/ [accessed December 4th 2018]

Young A.R., S.L. Walker, J.S. Kinley et al. (**1990**) Phototumorigenesis studies of 5-methoxypsoralen in bergamot oil: evaluation and modification of risk of human use in an albino mouse skin model. *Journal of Photochemistry and Photobiology B: Biology*, 7/2–4, 231-250.

Ziegler M., H. Brandauer, E. Ziegler, G. Ziegler. (**1991**) A different aging model for orange oil: deterioration products. *Journal of Essential Oil Research*, 3/4, 209-220. DOI:10.1080/10412905.1991.9697931