Investigations into heat- and light-induced terpene modifications in essential oils

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CONTENTS

1 PRELIMINARY REMARKS

The work presented in this doctoral thesis is based on a selection of papers published in international peer-reviewed journals. All scientific contributions to journals or congresses during the period of this thesis are compiled below in chronological order:

Full papers:

1. **H. Bitterling, P. Lorenz, W. Vetter, J. Conrad, D. R. Kammerer, F. C. Stintzing**; Rapid spectrophotometric method for assessing hydroperoxide formation from terpenes in essential oils upon oxidative conditions; *Journal of Agricultural and Food Chemistry* 68, (**2020**), 9576–9584.

2. **H. Bitterling, P. Lorenz, W. Vetter, D. R. Kammerer, F. C. Stintzing**; Storage-related changes of terpene constituents in caraway (*Carum carvi* L.) under real-time storage conditions; *Industrial Crops & Products* 170, (**2021**), 113782.

3. **H. Bitterling, P. Lorenz, W. Vetter, D. R. Kammerer, F. C. Stintzing**; Photo-protective effects of selected furocoumarins on *β*-pinene, *R*-(+)-limonene and *γ*-terpinene upon UV-A irradiation; *Journal of Photochemistry and Photobiology*, *A: Chemistry* 424, (**2021**), 113623.

4. **H. Bitterling, L. Mailänder, W. Vetter, D. R. Kammerer, F. C. Stintzing**; Photo-protective effects of furocoumarins on terpenes in lime, lemon and bergamot essential oils upon UV light irradiation; *European Food Research and Technology* 248, (**2022**), 1049-1057.

Further publications not included in the dissertation:

5. **H. Bitterling, U. Schäfer, G. Krammer, L. Meier, S. I. Brückner, B. Hartmann, J. Ongouta, R. Carle, C. B. Steingass**; Investigations into the natural occurrence of 1-phenylethyl acetate (styrallyl acetate); *Journal of Agricultural and Food Chemistry* 68, (**2020**), 8613–8620.

6. **L.K. Mailänder, P. Lorenz, H. Bitterling, F.C. Stintzing, R. Daniels, D.R. Kammerer**; Phytochemical Characterization of Chamomile (*Matricaria recutita* L.) Roots and Evaluation of Their Antioxidant and Antibacterial Potential, *Molecules* 27, (**2022**), 8508.

Oral presentations:

7. **H. Bitterling, P. Lorenz, W. Vetter, D. R. Kammerer, F. C. Stintzing**; Photo-protective effects of selected furocoumarins on *D*-(+)-limonene, *β*-pinene and *γ*-terpinene; 51st International Symposium on Essential Oils (**ISEO 2021**), (online), 11/12/2021 – 11/14/2021.

Poster presentations:

8. **H. Bitterling, P. Lorenz, W. Vetter, D. R. Kammerer, F. C. Stintzing**; Veränderungen im Terpenprofil von ätherischem Kümmelöl (*Aetheroleum Carvi*) bei Lagerung unter praxisnahen Bedingungen; 49. Deutscher Lebensmittelchemikertag, (online), 08/30/2021 – 09/01/2021; Lebensmittelchemie 75, (**2021**), S034.

2 AIM OF THIS WORK

Essential oils belong to the volatile secondary plant metabolites, having a lipophilic character and molecular weights below 300 Da. Essential oils are composed of compounds belonging to different chemical substance classes, whereby mono- and sesquiterpenes usually make up the main part of the oil. Due to their low boiling point, they were normally gained by hydro-distillation, except for citrus essential oils which are produced by mechanical peel extraction. In this latter process, further non-volatile plant compounds such as furocoumarins are also transferred into the essential oil fraction. Due to their pleasant smell and fine aroma, essential oils are widely used such as in food and beverages, as well as in cosmetics. Because of their distinct pharmacological properties, a considerable number of essential oils are also used in phytotherapy.

Essential oils are known to undergo several chemical changes during processing and storage, thereby losing their characteristic olfactory and medicinal properties. Essential oils tend to easily degrade in the presence of oxygen. Various factors including elevated temperature, light, and catalytic agents may accelerate degradation rates. The formation of oxidative reaction products, such as hydroperoxides, have been described in the scientific literature. In hydroperoxide quantitation, the commonly used iodometric titration poses several problems such as the requirement of high sample amounts and the impact of accompanying compounds in the respective sample.

Therefore, the first goal was to establish sensitive instrumental analytical methods for the compound-specific detection of terpene hydroperoxides and further oxidation products. These methods should be applied in several studies to gain more information on the alteration processes of terpenes and their corresponding essential oils on a molecular level. For this purpose, samples should be stored under real-time conditions including known essential oil plants such as caraway (*Carum carvi* L.) and lemon (*Citrus limon* (L.) Osbeck). Furthermore, a closer look at the impact of the surrounding matrix on terpene degradation should be taken. Not only the plant-like cell matrix but also non-terpenoid substances in the essential oil are to be examined more closely. The focus should be on furocoumarins that were removed and again added into expressed agrumen oils. Hereby, the differences in autoxidation and photo-oxidation should also be considered.

3 GENERAL INTRODUCTION

3.1 Essential oils – Historical aspects

Essential oil is the summarizing term for a liquid multicomponent mixture composed of volatile plant secondary metabolites of different chemical classes. Due to their pleasant smell they have been appreciated as natural remedies, flavors, and fragrances, and interests in their recovery already existed in ancient days [1,2]. The physico-chemical process of essential oil evaporation and re-condensation may be dated back to approximately 3500 BC based on an old apparatus used for distillation in Mesopotamia which is exhibited in the Taxila Museum in Pakistan today [3,4]. Similarly, in Egypt resins and essential oils containing various terpene compounds were used for the mummification processes of human and animal bodies. For instance, essential oils of the coniferous trees pine (*Pinaceae*), including cedar (*Cedrus* Trew), and juniper (*Juniperus* L.) were used because of their antifungal and antibacterial effects on the conservation of the mummy [5]. Arguably, similar applications are assumedly valid for all high cultures [1]. The first written evidence for the use of aromatic plants like anise (*Pimpinella anisum* L.*)*, fennel (*Foeniculum vulgare* Mill.*)*, thyme (*Thymus vulgaris* L.), and coriander (*Coriandrum sativum* L.*)* has been described in the so-called Papyrus Ebers, which is named after the German Egyptologist G. Ebers (1837 – 1898) [1]. The physician and alchemist Theophrastus Bombast of Hohenheim, named Paracelsus (1493 – 1541), developed various medicines and extracts from aromatic healing plants. He believed that by distillation the most valuable part of the plant could be separated [1]. The name *Quinta essentia* describes the “essential” part that is separated from the “non-essential” part of the plants. The name ´essential oil´ used today is dated back to Paracelsus of Hohenheim [1,6]. A systematic investigation of distillation was first conducted and further developed by the Strasbourg physicist Hieronimus Brunschwig in the sixteenth century. The process is described for different types of stills in his book “*Liber de arte distillandi de compositis*" at the example of the four essential oils of rosemary (*Rosmarinus officinalis* L.), spike lavender (*Lavandula latifolia* M.), juniper wood (*Juniperus* L.), and turpentine oil (*Pinus spp*.) [1,7]. He was followed by the German alchemist Johann Rudolph Glauber, who established further improvements in the field of distillation processes including improved fractionation by a rectification process [1,8]. However, the first scientific study on the ingredients of the extracted

essential oils was conducted much later in the nineteenth century and is attributed to the French chemist Jean Baptiste Dumas (1800 – 1884) who first characterized some hydrocarbons and oxygen-containing compounds [1].

The greatest systematic work on the composition of essential oils was performed by Otto Wallach (1847 – 1931) and August Kekulé (1829 – 1896). Fractionated distillation enabled the isolation of individual components that could then be further characterized and that were named terpenes, as they were first extracted from turpentine oil (named after Terpentin, the German name of turpentine) [1]. Also, Wallach stated the hypothesis that terpenes must be composed of isoprene units [9]. Since modern methods of structure elucidation were not yet available at that time and were based solely on spectroscopic methods, further physico-chemical characteristics such as boiling point, density, and optical behavior were used [1,9]. Further, thoughtful and elegantly planned degradation experiments generated additional structural information on terpenes. Wallach published more than 180 articles on terpene chemistry and was later honored with the Nobel Prize for Chemistry in 1910 [1,9]. In the following years, other well-known chemists such as Walter Hückel, Vladimir Prelog, and Robert Burns Woodward continued to advance the elucidation of terpene structures and their stereochemistry [1]. This was of course also due to the continuous improvement of analytical methods, primarily the pioneering work in the field of gas chromatography (GC) by Erika Cremer (1900 – 1996) [10]. In addition, sensitive nuclear magnetic resonance (NMR) spectroscopy was eventually applied to the structure elucidation of terpenes [1,10,11].

Today, essential oils are obtained by distillation (93%) or extraction (7%) of the corresponding plant material e.g., fruit peel, seeds, flowers, bark, and root, and their production quantity has constantly increased over the last years also boosted by a strong demand for natural ingredients in many fields of application (Figure 1) [12–14]. Large quantities of essential oils are produced for the food and beverage, cosmetic and fragrance, personal care and household, and health (aromatherapy and phytomedicine) industries [15]. Nowadays, the global market for essential oils is quantitatively dominated by orange (*Citrus sinensis* (L.) Osbeck) oil (51,000 t/a) and corn mint (*Mentha arvensis* L.) oil (32,000 t/a) (Figure 2, chapter 3.2) [16]. Some others are produced on a much smaller scale due to rarity resulting in very high prices like

rose essential oil (*Rosa* × *damascena* Mill.) 6,000-10,000 €/kg or agarwood oil (*Aquilaria malaccensis*) 6,000-11,000 €/kg [13].

Figure 1. Representative distillation apparatus as used to produce various essential oils. Here for rose essential oil production in Bulgaria. © Sonja Schrack-Belschner.

3.2 Industrial production of essential oils

The essential oils market is a global trade due to a growing demand in many fields (Figure 2) [17]. The predominant share of essential oils is currently generated by hydro-distillation (section 3.2.1). Furthermore, extraction with organic solvents (section 3.2.2) or direct expression of fruits or fruit peel is performed e.g., in the case of citrus fruits (section 3.2.3) [12,18]. Most important, the respective production method strongly determines the specific composition of the obtained essential oil [19]. In some cases, partial fractionation by vacuum distillation or solvent extraction is carried out as a subsequent processing step [16]. Hereby, unwanted compounds such as hydrocarbons or furocoumarins can be partly removed from the essential oil [16].

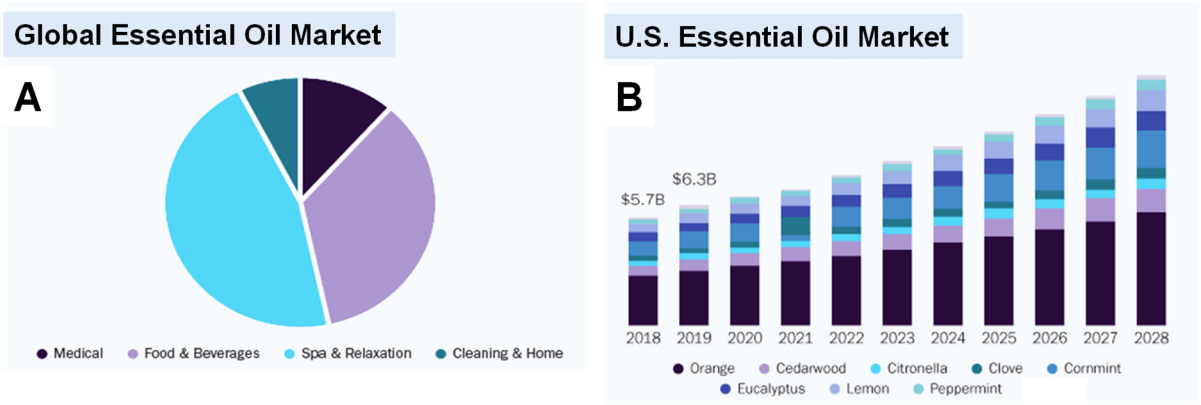

Figure 2. The global essential oil market is dominated by the cosmetic (spa & relaxation) and food and beverage markets (**A**). The future trend is moving towards further growth in the essential oil market as illustrated for the US (**B**). Production volumes from 2020 on are only estimated. Illustration adapted from the Grand View Research in 2022 [17].

3.2.1 Distillation

Distillation is certainly the oldest method used to gain essential oils from plant raw materials. In commercial production, mainly two different methods are applied, namely steam- and hydro-distillation, the latter of which was also used in the study presented in **paper 2** [16,20]. In a hydro-distillation apparatus (Figure 3), the whole plant material is covered by water and subsequently boiled for a given period [16]. With this simple method, the cell walls of the plants soak up water due to osmosis and then are disrupted by the expansion of the heated water [16]. As a consequence, the essential oil is released into the water phase where it is carried out by the water steam (Figure

3, left panel) [21]. In steam distillation, water vapor is produced in a separate device and fed directly into the pot stocked with dry plant material. Then, the volatile essential oil is leached and carried with the water vapor into the condenser. In the downstream so-called Florentine flask, the essential oil is separated from the water due to differences in density and polarity. Commercial still pots hold a volume of up to 5,000 L and are loaded with several hundred kilograms of plant material [16,21].

The underlying physico-chemical principle of both distillation methods is that the vapor pressure of biphasic liquid-liquid systems is the sum of the partial vapor pressures [22]. Therefore, the water-essential oil system boils at a lower temperature than pure water [16,22]. Still, the energy consumption of distillation processes is very high [23], and leads to high energy costs and carbon dioxide emissions from burning fossil fuels [24]. Hence, future applications should be evaluated in terms of sustainability considering among others alternatives to fossil fuels.

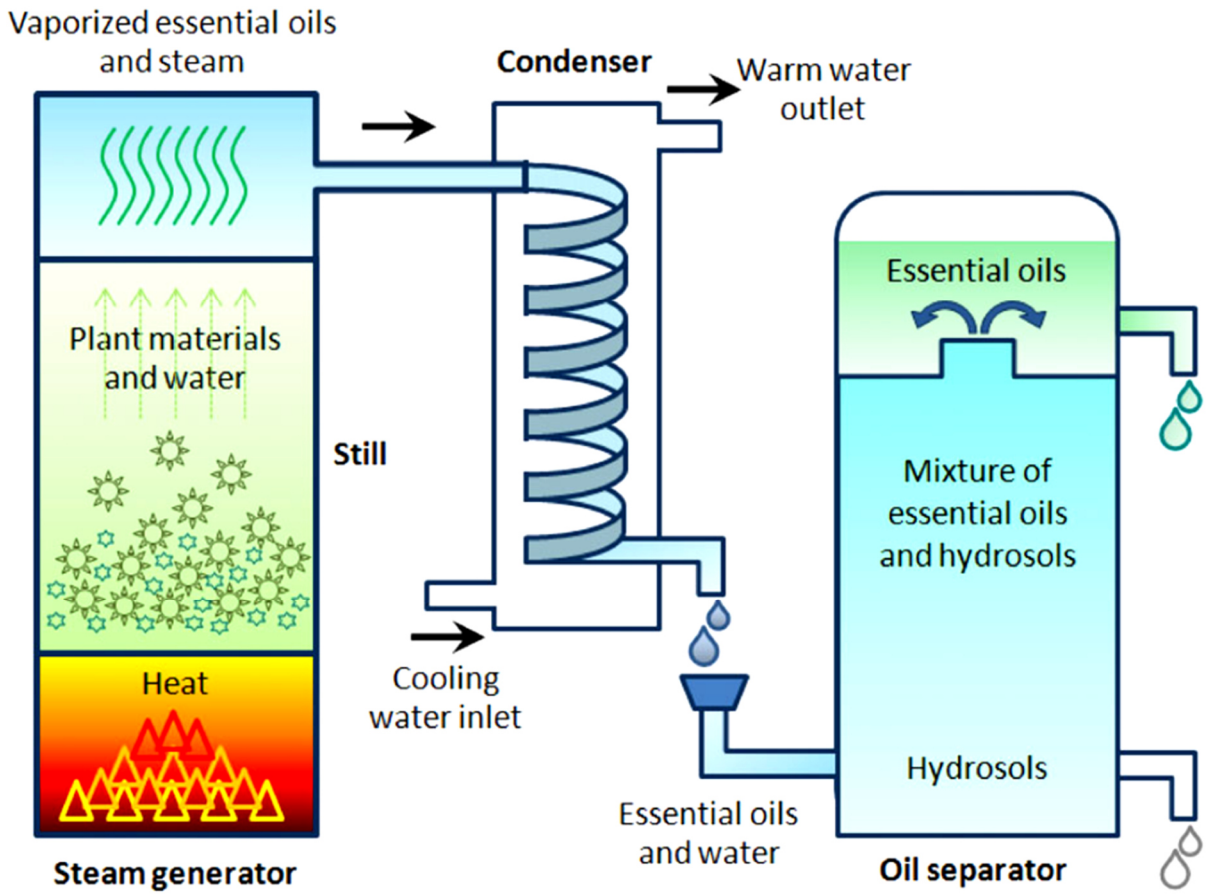

Figure 3. Technical illustration of a hydro-distillation apparatus for commercial essential oil production. Illustration adapted from Tongnuanchan *et al*. [21].

3.2.2 Solvent extraction

Essential oils can also be gained by extraction with organic solvents [25]. On an industrial scale, the method is preferably used for sensitive essential oil compounds in flowers and petals or to obtain high-quality flavor extracts [14,26]. Conventional extractions are mostly performed with *n*-hexane, petroleum ether, or dichloromethane, preferably applied to achieve good phase separation due to high differences in density compared to water [14]. The resulting extract, which is called oleoresin, does not only contain the essential oil but also all other compounds in the sample that are soluble in the organic solvent. As a consequence, extracts also contain non-volatile plant compounds like waxes [21,27–29]. Small-scale extractions are often carried out in a Soxhlet apparatus or a distillation-extraction apparatus according to Likens-Nickerson [29,30]. Supercritical fluid extraction (SFE) is a modern environmentally friendly and energy-efficient extraction method [31,32]. Hereby, supercritical carbon dioxide (CO_2) is frequently used for extraction due to its well-suited critical point at 304 K and 7.4 MPa [19]. Extraction with supercritical carbon dioxide is highly efficient for non-polar volatile compounds as it has a low viscosity and a high diffusivity [27]. In addition, CO_2 is non-toxic and it can be completely removed from the extract by pressure reduction [21,27]. Supercritical CO_2 has also been used to fractionate flower essential oils obtained by organic solvent extraction (a.k.a. flower concretes) [33]. Flower concretes, as those produced from rose flowers, often contain high shares of waxes which can be removed by SFE [34]. Also, SFE with supercritical water proved to be suited for the extraction of essential oils, but this method is less frequently applied because it requires harsh physical conditions [21,34]. Separation of essential oils from co-extracted compounds (e.g., waxes) in the extract (a.k.a. *essences concrètes*) is traditionally obtained by a subsequent extraction step with ethanol to obtain *essences absolutes* [30]. Also, *enfleurage* describes such a historical preparation method by application of beef tallow and pork fat [35]. In this process, the plant material is placed on glass plates coated with these fats for some hours or days so that the lipophilic plant compounds diffuse into them. The essential components are then extracted from the fat using alcohol [35,36]. Today, this very gentle extraction method for essential oils is still being used for the production of very high-priced and sensitive flower oils such as those of rose and jasmine (*Jasminum officinale* L.) [28,30].

3.2.3 Direct extraction (expression)

The direct extraction of essential oils represents a physical process of cold-pressing or expression and is mainly used to gain essential agrumen (citrus) oils located in the fruit peel [14]. Four different processing techniques are commonly used, i.e. pellatrice, sfumatrice, the Brown Oil Extractor (BOE), and the Food Machinery Corporation Inline Extractor (FMC) [14,37]. By means of pellatrice (i.e. use of a peeling machine) and BOE extractors, essential oils are directly obtained by puncture from the peel of the fruit followed by the separation of impurities. By contrast, sfumatrice and FMC extractor techniques produce the essential oil from the whole fruit. In the first step, the juice and essential oil is extracted followed by the separation of the resulting oil-juice emulsion by centrifugation [14,18]. Best sensory qualities are achieved in cold-pressed essential citrus oils due to the avoidance of heat which reduces the oxidation of sensitive citrus terpenoids like limonene, neral, geranial, and citronellal [16]. Also, the essential oils used in the study presented in **paper 4** were obtained by direct expression. The highest production volumes are described for orange, lemon, grapefruit (*Citrus* × *paradisi*), and mandarin (*Citrus reticulata*) essential oils. The cold-processed essential oils may be cooled (“wintered”) to precipitate co-occurring waxes. Subsequently, expressed peels can be treated with hot steam to recover the remaining limonene for use in the solvent industry [14,16].

3.3 Chemical constituents of essential oils

Two different classes of metabolites are produced by plants and are categorized as follows. Those that are crucial for the survival of cells and reproduction are termed primary metabolites, while the secondary ones are indispensable for the unsupportive defense of any type of attack (herbivores, microbes, weeds) or stress (UV light) on the plant [37–39]. All components that number among essential oils belong to the class of secondary metabolites [37]. The most relevant essential oil compound classes will be described in the following. Namely, mono- and sesquiterpenes (sections 3.3.1 and 3.3.2) represent the largest class of volatile organic compounds in plants [40]. Moreover, non-volatile phenylpropanoids (section 3.3.3) and furocoumarins (section 3.3.4) are two further important substance classes found in various essential oils [41,42]. Additional minor components such as paraffins and long chain secondary alcohols that belong to the co-extracted waxy fraction are mainly found in flower concretes due to their presence on the surface of rose petals for instance (sections 3.3.5) [43].

3.3.1 Monoterpenes

Monoterpenes are produced in the secondary metabolism of various plant species as well as in insects and microorganisms [44–46]. They are generated by linking two isoprene (C_5, hemiterpenes) units which results in a variety of structural C_{10} isomers [47]. The building principle is described by the so-called isoprene rule that is based on the work of Otto Wallach (Nobel Prize 1910) and subsequent findings of Leopold Ruzicka (Nobel Prize 1939) [48]. It states that terpenoid substances are formed from a different number of single isoprene units. The initial biosynthesis of terpenoids starts with three acetyl-CoA units and results in mevalonic acid (mevalonate pathway). This central precursor is used to form the isoprene unit isopentenylpyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) via mevalonic acid diphosphate. Catalyzed by geranyl pyrophosphate synthase (GPPS) and farnesyl pyrophosphate synthase (FPPS), the condensation of IPP and DMAPP results in monoterpenes and sesquiterpenes, respectively (Figure 4) [40,49,50]. In plants, the reaction is catalyzed by terpene synthases in the cytoplasm of the cell [49].

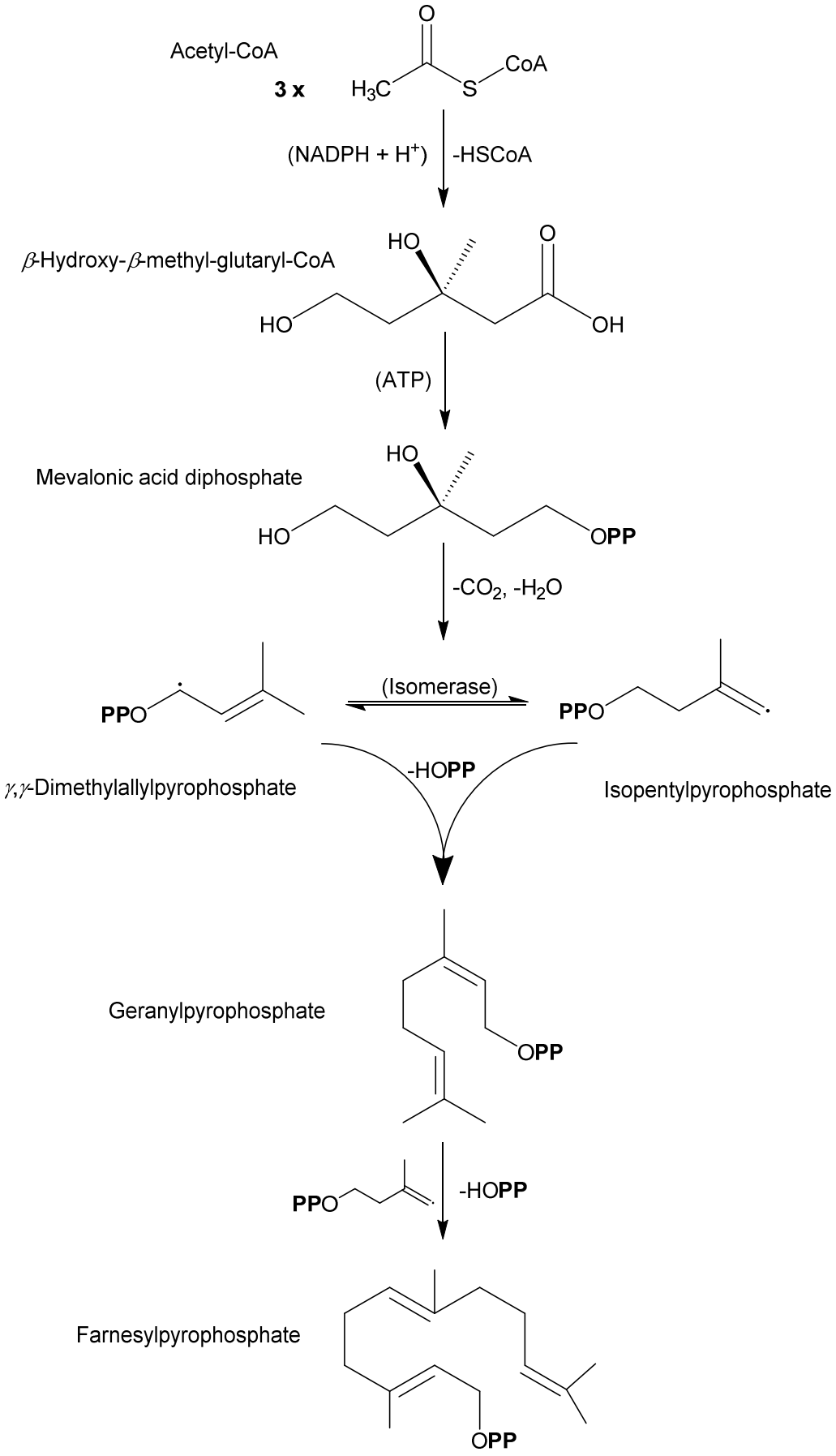

Figure 4. Biosynthesis of monoterpenes and sesquiterpenes. Illustration adapted from Breitmaier [50].

Here, the term terpene is mostly used representatively for monoterpenes. Whereby, narrowly defined, (mono)terpenes are hydrocarbons that exclusively consist of carbon and hydrogen atoms. Well-known representatives are for instance *R*-(+)-limonene, α-pinene and γ-terpinene (Figure 5; **1**, **2**, **3**), which were used for oxidation and irradiation experiments in the studies presented in **papers 1-3**. For limonene, the stereocenter is exemplarily labeled with an asterisk (Figure 5, **1**). This carbon atom bears four different groups or atoms which leads to the possible existence of two enantiomers [37,51]. Enantiomers show the same physico-chemical properties but frequently differ in their smell.

For instance, *S*-(+)-carvone is attributed with a distinct caraway odor, while *R*-(-)-carvone has a spearmint like odor [52]. Monoterpenes that additionally feature oxygen within different functional groups are classified as terpenoids. As illustrated in Figure 5, terpenoids can be further divided into alcohols (e.g., carveol, **4**; citronellol, **5**), ketones (e.g., carvone, **6**; menthone, **7**), esters (e.g., linalyl acetate, **8**), aldehydes (e.g., citral, **9**), and oxides (e.g., 1,8-cineol, **10**) [3,53,54]. Terpenes and terpenoids represent the biggest class of natural compounds and more than 30,000 individual substances are known [51]. Due to their high vapor pressures, they can be found in the atmosphere as so-called biogenic volatile organic compounds (BVOCs) [55,56].

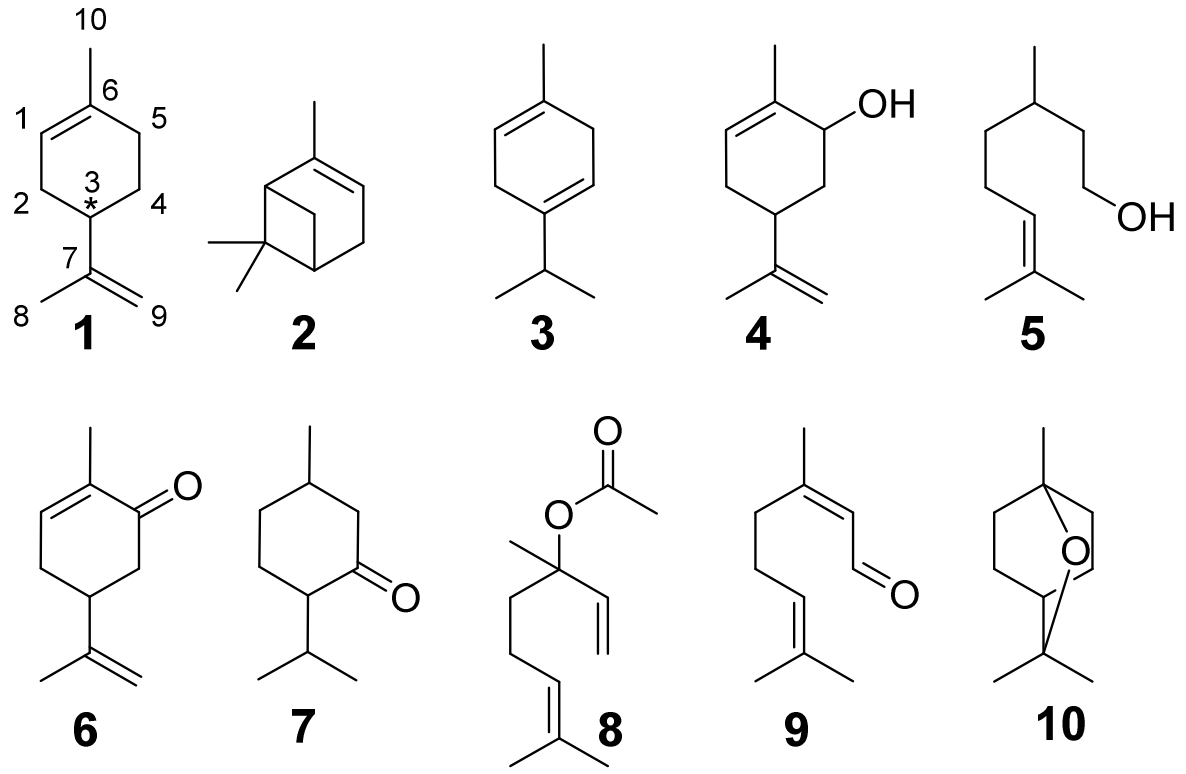

Figure 5. Chemical structures of limonene (**1**), α-pinene (**2**), γ-terpinene (**3**), carveol (**4**), citronellol (**5**), carvone (**6**), menthone (**7**), linalyl acetate (**8**), citral (*cis*-form: neral, *trans*-form: geranial, **9**), and 1,8-cineol (**10**). The stereocenter is exemplarily labelled in **1**.

3.3.2 Sesquiterpenes

Similar to monoterpenes (section 3.3.1), the biosynthesis of sesquiterpenes is catalyzed by farnesyl pyrophosphate synthase and results in acyclic and cyclic compounds containing 15 carbon atoms with farnesol being the ubiquitous sesquiterpene precursor [49,51]. Typically, the higher molecular weight of sesquiterpenes is linked with higher boiling points and a lower volatility compared to monoterpenes [57]. Thus, they often contribute less to the odor profile of essential oils [36]. However, sesquiterpenes often show good fixing properties and contribute to the base note of essential oils and fragrance mixtures [36,58]. Structurally, sesquiterpenes are much more varied than monoterpenes due to the presence of three double bonds in the general precursor molecule farnesyl pyrophosphate [59,60]. Well-known representatives are for instance *β*-caryophyllene (Figure 6; **11**) that contributes with around 15% to clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) essential oil [61] while valencene (Figure 6; **12**) is present in orange essential oils [62] and is used for the synthesis of nootkatone (Figure 6; **13**). The latter is a high priced sesquiterpene

that dominates the aroma of grapefruit and finds a wide range of applications in the flavor and fragrance industry [63].

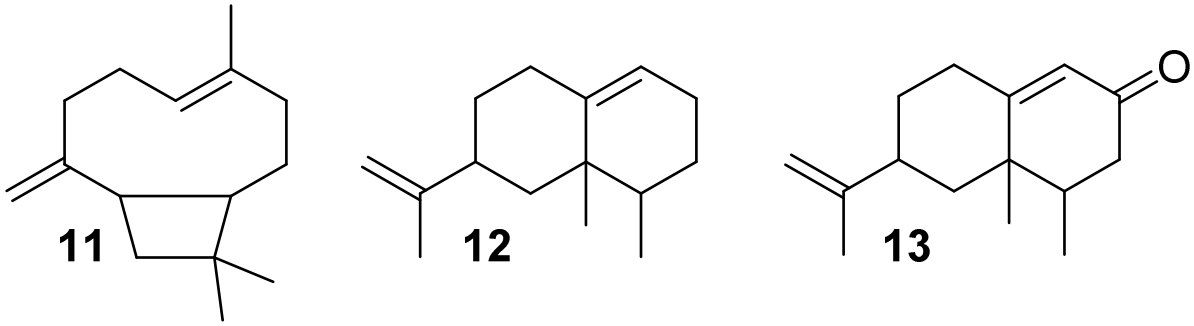

Figure 6. Chemical structures of the sesquiterpenes *β*-caryophyllene (**11**), valencene (**12**), and nootkatone (**13**).

3.3.3 Phenylpropanoids

Phenylpropanoids are a further group of organic volatile compounds found in essential oils [37,42]. These secondary plant metabolites show protective effects against herbivores, bacterial infections, and UV light [42]. In addition, they were found to act as phytoalexins [64]. The biosynthesis of phenylpropanoids is based on the modification of phenylalanine. This aromatic amino acid is modified by the shikimate pathway, into which approximately 30% of the photosynthetically fixed carbon enters [42,65]. Phenylalanine is converted into cinnamic acid and further transferred to various phenylpropanoids such as eugenol (Figure 7; **14**), that contributes to more than 50% of clove essential oils, and safrole (Figure 7; **15**) which is abundant in sassafras (*Sassafras albidum* J.Presel) root bark [61,66]. However, it has to be mentioned that strong mutagenic and cancerogenic effects have been described for some phenylpropanoids including methyl eugenol (Figure 7; **16**) which is present in small amounts in rose essential oil [67].

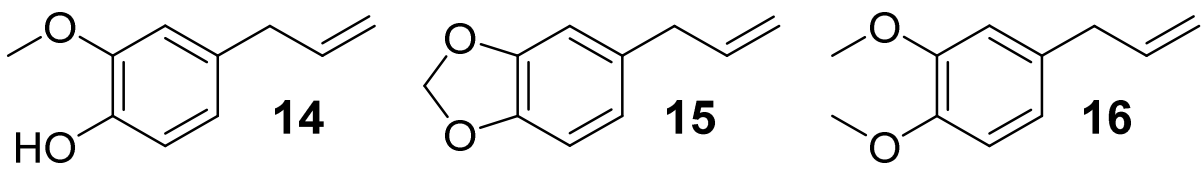

Figure 7. Chemical structures of the phenylpropanoids eugenol (**14**), safrole (**15**) and methyl eugenol (**16**).

3.3.4 Furocoumarins

Furocoumarins are a further compound class present in some essential oils, especially in those obtained from the *Apiaceae* and *Rutaceae* families [68]. Furocoumarins are both tricyclic and heterocyclic compounds that can be structurally divided into linear psoralens (furan ring fused at position 6,7 to coumarin; Figure 8, **17**) and non-linear angelicins (furan ring fused at position 7,8; Figure 8, **18**) [69,70]. In plants, psoralen and angelicin biosynthesis starts with umbelliferone (7-hydroxycoumarin) as the ubiquitous precursor. More than 50 furocoumarins have been discovered in plants yet, whereby the linear psoralens represent the dominant group in all known cases [69].

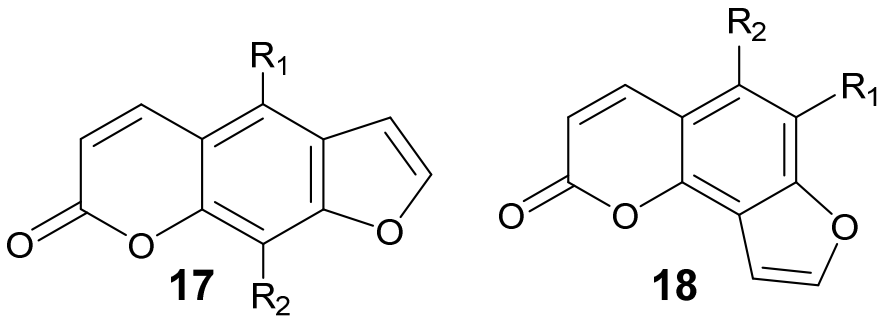

Figure 8. Basic structures of linear-type psoralens (**17**) and the angular-type angelicins (**18**) containing several functional side groups (R_1 and R_2, see Table 1).

The *Rutaceae* family includes all citrus fruits which play an important role in essential oil production. The process of essential oil extraction takes place during juice production. The essential oil is recovered by peel abrasion or from expressed juices (section 1.2.3). As these cold-pressed essential oils have not undergone a distillation process, the non-volatile furocoumarins are gained together with the true volatile essential oil constituents, to which they can contribute with up to 15 weight-% [18]. Well-known citrus furocoumarins are oxypeucedanin, isopimpinellin, bergapten, and bergamottin [71]. The latter two compounds as well as xanthotoxin and bergaptol were used in photo-oxidation experiments in the study presented in **paper 3**. Diverse coumarin derivatives are also found in cold-pressed essential agrumen oils [18]. In 1933, the first structure of a furocoumarin, i.e. xanthotoxin, was elucidated for the first time by Späth and Holzen, after its isolation from *Fagara xanthoxyloides* L. [71,72]. Furocoumarins act as natural pesticides in plants that can inhibit microbial growth and

the attack of herbivores [73,74]. Their mode of action is based on activation by UV-A light due to a strong photo-reactivity. Therefore, contact of animals with furocoumarins on the skin can lead to the so-called photodermatitis with sunburn-like skin injuries and heavy blisters [75]. *Vice versa*, furocoumarins are used to treat skin diseases such as vitiligo and psoriasis in PUVA (psoralen + UV-A) therapies [75]. Photo-activation (absorption) of furocoumarins takes place in a wavelength range of 320 - 380 nm and leads to the activated singlet state (S_1, Figure 9). After fast internal conversion (IC), this short-lived state can be either deactivated by visible fluorescence light emission (> 380 nm) back to S_0 or by intersystem crossing (ISC) which leads to the triplet state T_1 (Figure 9). The triplet state can be deactivated by phosphorescence light emission at even higher wavelengths (Figure 9) [71].

Table 1. Substitution patterns of representative furocoumarins based on the structures illustrated in Figure 8.

Backbone	R_1	R_2	Trivial Name
17	OCH_3	H	Bergapten
	H	OCH_3	Xanthotoxin
	OH	H	Bergaptol
	$OC_{10}H_{17}$	H	Bergamottin
18	H	H	Angelicin
	H	OCH_3	Isobergapten

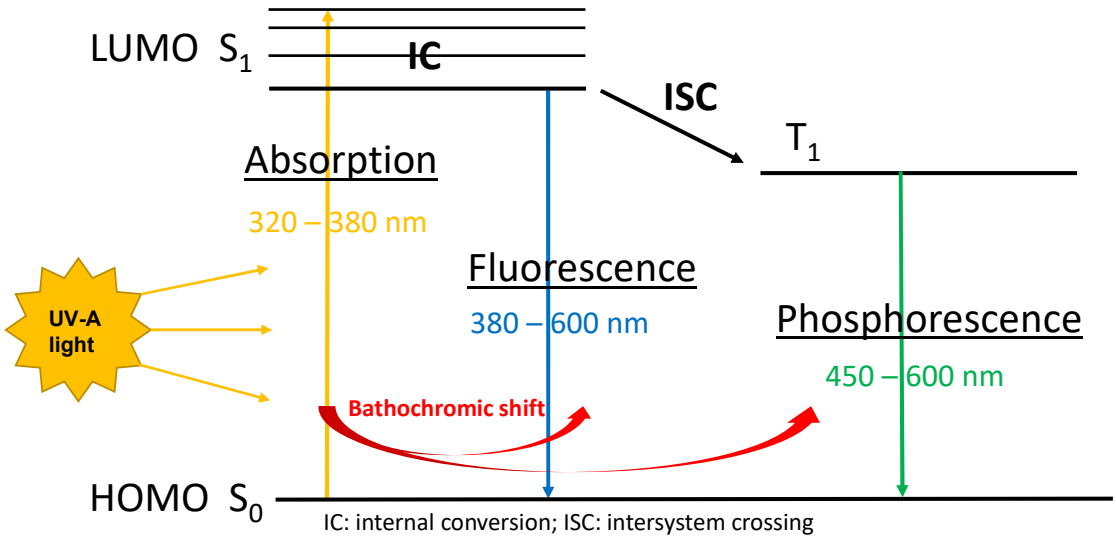

Figure 9. Jablonski diagram showing the activation of furocoumarins by UV-A light followed by the emission of energy by fluorescence and phosphorescence after internal conversion (**IC**) and intersystem crossing (**ISC**) [71].

3.3.5 Other constituents

Apart from commonly known compounds described before, further substances may be present in essential oils. For instance, wax-derived linear hydrocarbons (*n*-alkanes) were described in rose essential oils that were transferred into the oil by distillation of the petals [76,77]. The cuticular wax on the leaves and petals represents a natural barrier against environmental impacts and protects against water loss and uncontrolled gas exchange [76]. The wax mainly composed of linear hydrocarbons (*n*-alkanes) as well as long-chain primary and secondary alcohols and can be transferred into the essential oil by distillation or extraction [43,78]. The hydrocarbon fraction can contribute to more than 20% of rose essential oil, with the highest shares originating from nonadecane ($C_{19}H_{40}$; Figure 10, **20**) and heneicosane ($C_{21}H_{44}$) [77,79,80]. In addition, several long-chain carboxylic (fatty) acids and their ester derivatives have been detected in fractions of rose concrete. These fatty acids were esterified with linear alcohols and terpenols [26,81]. As described by Micali *et al*. (1990), *n*-alkanes were also detected in cold-pressed lemon, bergamot (*Citrus bergamia*), and orange oils as well as in oregano (*Origanum vulgare* L.) essential oil [82,83]. In addition, polymethoxyflavones such as tangeretin (Figure 10, **19**) have been described in essential agrumen oils [18]. In general, cold-pressed essential oils represent a quite complex mixture as they contain both volatile and non-volatile substances due to the lacking distillation that represents a further separation step [14,37].

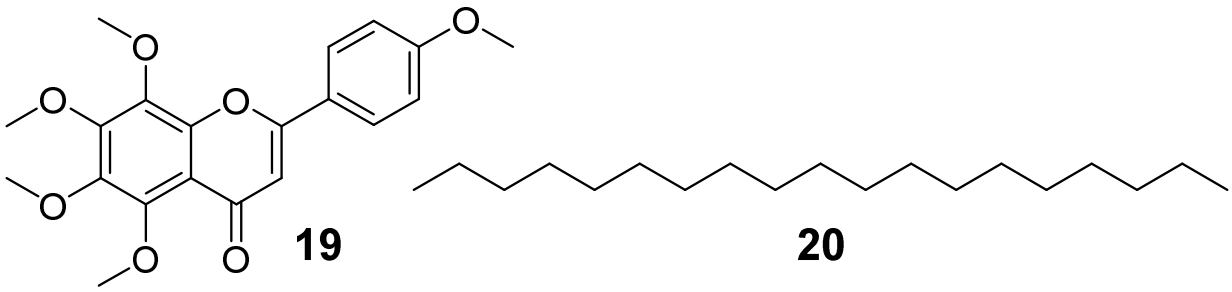

Figure 10. Tangeretin (**19**), found in citrus essential oils, and nonadecane ($C_{19}H_{40}$**, 20**), found in rose essential oils.

3.4 Biochemical aspects of essential oils in nature

At the beginning of volatile organic compounds research, it had been a mystery why plants are synthesizing and emitting these mostly fragrant small molecules. In 1934, however, ethylene the first signalling molecule emitted by plants was described. The latter has an enormous physiological effect on the ripening of fruits and leaves [39]. Essential oils are part of the natural volatile organic compounds (NVOCs) that include all lipophilic low molecular weight compounds (≤ 300 Da) with low boiling points and high vapor pressures [84–86]. NVOCs are predominantly emitted from flowers and vegetative plant parts, but are also found in roots [85]. In flowers, they are biosynthesized in epidermal cells, from which they can be easily released into the air [87]. In vegetative plant material, the volatiles are produced in specialized ducts and stored in oil cells from which they may be released after mechanical disruption of the plant tissue e.g., upon attack by herbivores. For example, the essential oil of scented pelargonium or sage is stored in a sac at the end of the external glandular hairs to be easily released into the atmosphere (Figure 11) [38,87].

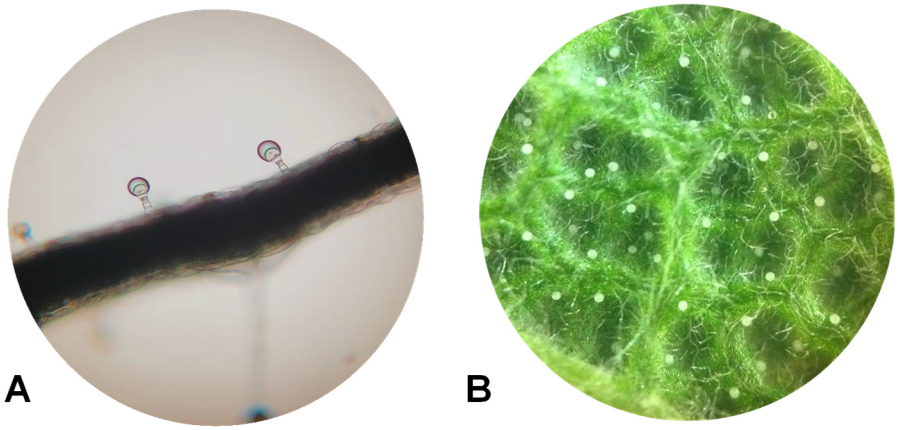

Figure 11. Oil ducts and glandular hairs of scented pelargonium (*Pelargonium citrodorum* L.) (**A**, cross section, 400× magnification) and sage (*Salvia officinalis* L.) (**B**, 100× magnification). © Angela Mann.

Different studies eventually revealed numerous biological functions of NVOCs emitted by plants. These include defence against herbivores and phytopathogens, attraction of pollinators, and uses as signalling molecules in plant communication (Figure 12) [38,86]. For instance, the herbivore attack on maize plants is indirectly defended by the release of β-caryophyllene which in turn stimulates nematodes that fight the corn

rootworm [88]. Moreover, NVOCs can also act as wound sealers and they feature antimicrobial activities [85,89]. Also, allelopathic effects were attributed to some essential oil volatiles, as decreased germination was found for weed seeds due to the presence of carvacrol and thymol [90]. Furthermore, the application of (*S*)-(+)-carvone effectively inhibited the sprouting of potatoes, which may be used as an ecological alternative to chemical sprout inhibitors [52,91]. In addition, also furocoumarins, predominantly present in cold-pressed agrumen oils, serve as defence agents of the plants against fungi, bacteria and insects [75]. Due to the photoactivation of furocoumarins by UV-A light (Figure 9) the microbial growth is inhibited and the mortality of herbivores is increased by their reaction with the DNA of the predators [72,75].

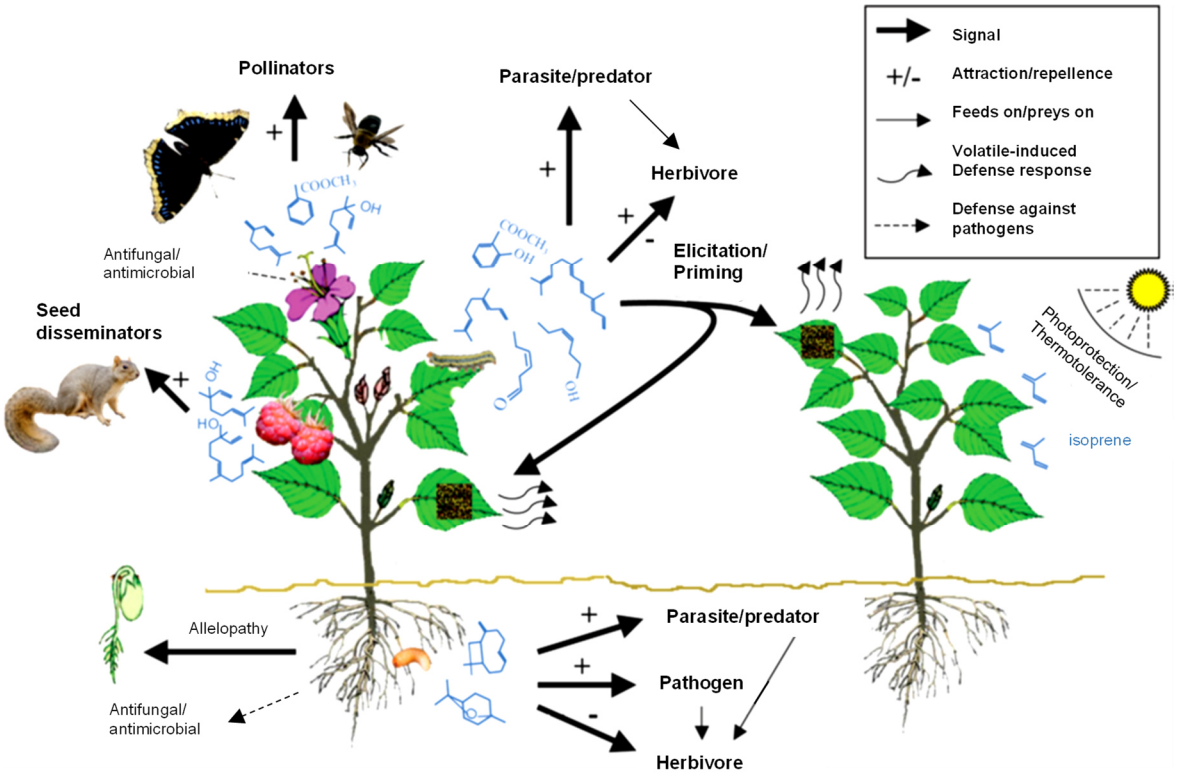

Figure 12. Typical plant volatiles and their effects on plant and environment. Adapted from Dudareva *et al*. (2006) [38].

3.5 Use of essential oils

The use of essential oils has a long tradition and finds application in many different fields of daily life [92]. The production of flavors and fragrances is not only managed by large, globally active companies with turnovers of several billion dollars, but also in the hands of small-scale manufacturers who may deal with exclusive and rare essential oils of unique scent [16]. The annual production volume of frequently used essential oils can amount to several thousand tons. Regarding production volume, orange (51,000 t/a) and corn mint (32,000 t/a) are currently the dominating essential oils, followed by lemon (9,200 t/a), eucalyptus (*Eucalyptu*s L'Her, 4,000 t/a), and peppermint (*Mentha* × *piperita* L., 3,300 t/a) (see Figure 2, chapter 3.2) [16,17].

3.5.1 Food and beverages

Herbs, seeds, blossoms, and spices have been used for ancient times in food preparation even though their contribution to nourishment is relatively low [54]. Yet, seasoning of meals shall improve taste, smell and digestion and additionally increase shelf-life. Seasoning traditionally belongs to culinary art [3,54]. Additionally, herbs and spices containing essential oils are valued due to the presence of "active compounds" with health-promoting effects [93]. Herbs and essential oils traditionally used for flavoring food and beverages include basil (*Ocimum basilicum* L.*)*, coriander, oregano, rosemary, thyme, sage (*Salvia officinalis* L.), and caraway (*Carum carvi* L.) [94,95]. Sage was described as one of the first spices used in food preparations including bread, cheese, desserts, meat, and fish, as well as alcoholic drinks [54,96,97]. The first documented application of essential oils for beverages was described in 1833 in a carbonated lemon soda drink [92]. Up till now, the majority of essential oils is used in soft drinks [3]. Hereby, the poorly water-soluble essential citrus oils need to be emulsified and stabilized by hydrocolloids like gum arabic or modified starches to ensure a pleasant fruity aroma of the final beverages [98]. Nowadays, essential oils are not only used because of their flavoring effects, but also because of their favorable techno-functional properties [99]. Due to their antioxidant and antimicrobial activity against gram-positive and gram-negative bacteria as well as fungi, they may substitute synthetic preservative agents in convenience food. For instance, carvacrol, that is a major compound of oregano and thyme essential oil, showed promising results due to disintegrational effects on the outer bacterial membrane [21]. Remarkable antimicrobial effects and insecticidal properties were also observed for citrus oils [99].

Hereby, spoilage of high-quality foods may be delayed due to an increased shelf-life. In addition, citrus oils were found to be active against larvae of the house fly (*Musca domestica*) or the tomato borer (*Tuta absoluta*) [99]. Furthermore, the protective function of bio-based packaging materials was improved by the incorporation of essential oils [100]. Nano-encapsulation of essential oils in packaging materials also showed promising antimicrobial effects without negative sensory impact on food [21,99].

3.5.2 Cosmetics

Fragrance plays a crucial role in cosmetic products as it is seen as the main factor for consumer purchase [101]. Accordingly, more than 30% of the industrially used essential oils are applied in the cosmetics and fragrance industry. Despite the availability of various synthetic fragrances, the demand for essential oils is still high [101,102]. Rose essential oil, also called the liquid gold because its high price of more than 10,000 €/kg, along with tea tree (*Melaleuca alternifolia* Cheel) and lavender (*Lavandula angustifolia* Mill.) are frequently used essential oils in cosmetics [102]. Also, the increased green consumerism in connection with the term “natural” ensures a large growth in the area of natural essential oils [101]. In cosmetics, essential oils are not only used due to their pleasant smell, but also because of their preservative effects which originate from their antimicrobial and antifungal properties [101,103]. Especially in natural cosmetics essential oils may thus replace synthetic ingredients such as parabens [101,102]. In addition, beneficial effects of essential oils have been exploited in the so-called cosmeceuticals. This term summarizes cosmetics that exert additional positive effects on the skin which may range from anti-aging, sun protection, and anti-acne effects to improvement of skin elasticity and firmness [101,104]. Distinct effects have been attributed to citrus essential oils from orange and lemon [101].

Furthermore, essential oils are also used in perfumes that often consist of mixtures of various different essential oils or components thereof diluted with alcohol [101]. Typically, the fragrances are subdivided into top, middle and base notes according to their volatility and diffusion into the air [36]. The scent of the light and highly volatile compounds of the top note lasts only for up to 10 min, while the scent of the middle, so-called “heart note” can be perceived for up to 1 h. While the first category is represented by citrus-type notes, the latter is dominated by spicy and floral notes such as from geranium (*Pelargonium graveolens* L’Hér. ex Aiton), lavender, or glove.

Heavy, low-volatile essential oil components from vanilla (*Vanilla planifolia* Mill.) or sandalwood (*Santalum album* L.) dominate the base note and provide a long-lasting depth to the perfume [101]. The “back to nature trend” also takes hold in the perfume area and high price essential oils such as agarwood as well as other flavoring naturals such as musk, amber, and civet are used to create appealing fragrances [3,101].

3.5.3 Phytopharmaceuticals

The use of essential oils for disease treatment and prevention has been described since ancient Egypt [2]. Further on, the Greeks and Romans used essential oils for aromatherapy in bath infusions and for inhalation [105,106]. Fired by drug resistance of bacterial pathogens, the limited availability of antibiotics, and several side effects noticed with synthetic pharmaceuticals, the call for alternative medicines is again on the rise [103,105]. Different essential oils have a long tradition in folk medicine for the treatment of pain and inflammation, viral and bacterial diseases, as well as antioxidant and insect repellent activities [103,107]. For instance, strong antibacterial effects were reported for the essential oils of thyme, oregano, tea tree, clove, coriander, lemon grass (*Cymbopogon citratus* (DC.) Stapf), and cinnamon (*Cinnamomum verum* J. Presl). Specifically, thyme and oregano essential oils were highly active against *Bacillus cereus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Escherichia coli* [103,108]. These effects were mainly attributed to the terpenoid components thymol and carvacrol whereby the destabilization of the bacterial cell membrane is the primary mode of action [103,105]. Furthermore, distinct antiviral effects against the herpes simplex virus were observed for eucalyptus and thyme essential oils due to their inhibition of viral replication [103,109]. Likewise, promising antifungal activities against *Candida* spp. and *Aspergillus* spp. may be helpful for immunocompromised patients [103]. In addition, essential oils may increase the skin penetration of transdermally administered drugs [103,110].

Free radicals and reactive oxygen species (ROS) formed by oxidative stress are known to cause damage to biological substances [111]. Secondary diseases such as accelerated aging, cancer, arthritis, inflammation, and diabetes can be the consequence of such chronic diseases. The use of essential oil compounds many of which are natural antioxidants could thus serve as a good prevention [105]. Furthermore, the positive effects of essential caraway constituents against various digestive disorders are well-known in folk medicine [97]. As an example, caraway is

used as infusions, added to food, or as sugar syrup to treat flatulence, constipation, and nausea [97]. Also, the ethno-botanical use of essential oil-bearing plants is reported against biting insects and mosquitos [107,110]. Nowadays, essential oils are still applied in malaria prophylaxis, as various compounds thereof showed a good repellent effect against the malaria mosquito (*Anopheles* Meigen) [110]. In addition, furocoumarins - typical for agrumen essential oils such as lime and lemon - are frequently reported in the therapy of skin diseases such as vitiligo and psoriasis [71] and in PUVA (psoralen + UV-A) therapy (see also section 3.3.4) [75]. In future, more investigations should be carried out to test further essential oils on their bioactivity profile and to better understand their pharmacodynamics [110]. In addition, there is a high demand for new antibiotics because of a rising resistance of bacteria against frequently used substances [103].

3.5.4 Personal care and household

The history of scented care and household products started in 1806, when William Colgate began to sell soaps and candles in New York [92]. Subsequently, companies like Palmolive and Procter & Gamble introduced the first toothpaste and hair care products that were pleasantly scented with essential oils [92]. Today, essential oils are used in many ways in personal care products and toiletries. For instance, spearmint (*Mentha spicata* L.), peppermint, or caraway essential oils are added to toothpaste and mouthwashes because of their fresh taste and cooling effect [92,112]. Furthermore, oregano and cinnamon essential oils have been added to teeth care products because of their antibacterial effects against the strong cariogenic bacterium *Streptococcus mutans* [113]. Products such as soaps, deodorants, and body cleansing products are typically scented with essential oils to increase consumer acceptance. The pleasant smell of essential oils, mixtures thereof, and single-flavoring terpene compounds is also utilized in household, bath, and kitchen cleaners [92,114]. An investigative study on commercial cleaning products indicated that essential oils or components thereof may amount up to 5% (*w*/*w*), with limonene and linalool being the most relevant ones due to the widespread use of orange, lemon, and lavender essential oils [115]. Last but not least, washing powders are hardly marketed without fragrant scents [114].

3.5.5 Agriculture

The massive use of pesticides in the agricultural industry since the 1940s has partly been linked with severe negative side effects such as toxicity against humans and animals, environmental pollution, or the spread of resistance [116–118]. In the search of environmentally friendly alternatives, essential oils may be of particular interest due to their antimicrobial, pesticidal, and herbicidal properties [119]. Promising inhibiting effects on many insects like beetles and the caterpillar larvae were observed upon application of rosemary essential oil [120]. Furthermore, eugenol from clove essential oil showed good acaricidal activities, while essential oils rich in limonene displayed strong antibacterial activity against gram-positive and gram-negative bacteria [107,121]. Also, antifungal effects were observed for cardamom essential oil, while lemongrass essential oil displayed antiviral effects against the tobacco mosaic virus [107,122]. Similarly, eucalyptus essential oil successfully suppressed the germination of weed and could thus serve as a bioherbicide [123]. Also, in post-harvest storage, the use of essential oils still represents a promising field of application. For instance, carvone from caraway essential oil was found to be an excellent sprout inhibitor in potatoes (*Solanum tuberosum* L.), and may replace toxic pesticides in long-term storage [97]. In the future, more research should be performed to verify the manifold perspectives of essential oils in the agricultural sector [119].

3.5.6 Conclusion

As was shown in the previous sections, the use of essential oils and their constituents is indispensable in our daily lives. New products at all levels, from food and beverages, personal care and household products to agriculture could benefit from new applications of these volatile secondary plant compounds.

3.6 Alterations of terpene constituents in the presence of oxygen

Despite the wide application and various additional perspectives for the industrial utilization of essential oils (section 3.5), it should be noted that the corresponding products are composed of a number of labile constituents [102,124–126]. Currently, essential oils are extracted from the plant material, thus losing their protective surroundings. Exposed to extrinsic factors, this may lead to chemical changes of individual oil compounds which often goes along with a loss of quality [2]. For instance, the monoterpene limonene (**1**) can be degraded by photo- and autoxidation (Figure 13). Photo-induced oxidation reactions predominately lead to different limonene hydroperoxides (**21**, **22**). In contrast, hydroperoxides and dioxetanes (**23**) formed via autoxidation were reported to further react to downstream products such as epoxides (**24**) and ketones (**6**) [2,127–130]. However, a clear distinction between photo-oxidation and autoxidation processes under real storage conditions is difficult, as they often prevail simultaneously [131]. Metal-catalyzed oxidation is particularly relevant during the contact of terpenes and essential oils with distillation apparatus and storage containers made of metal [16,132–134]. Hereby, diepoxides (**29**) and alcohols such as carveol (**4**) were identified as the main products resulting from catalytic limonene oxidation [132,135]. Enzymatic oxidation, as occurs specifically during biosynthesis or upon microbial spoilage, predominantly leads to products such as perillyl alcohol (**25**) and perillaldehyde (**26**) [136,137]. Technical oxidation of terpenes with ozone and related reactive molecules only plays a minor role [138]. However, this is an important reaction in atmospheric chemistry. Laboratory experiments confirmed that large parts of the volatile terpenoids emitted by plants are oxidatively degraded to follow-up products such as limonene aldehydes (**27**) and keto-limonene aldehydes (**28**) [138].

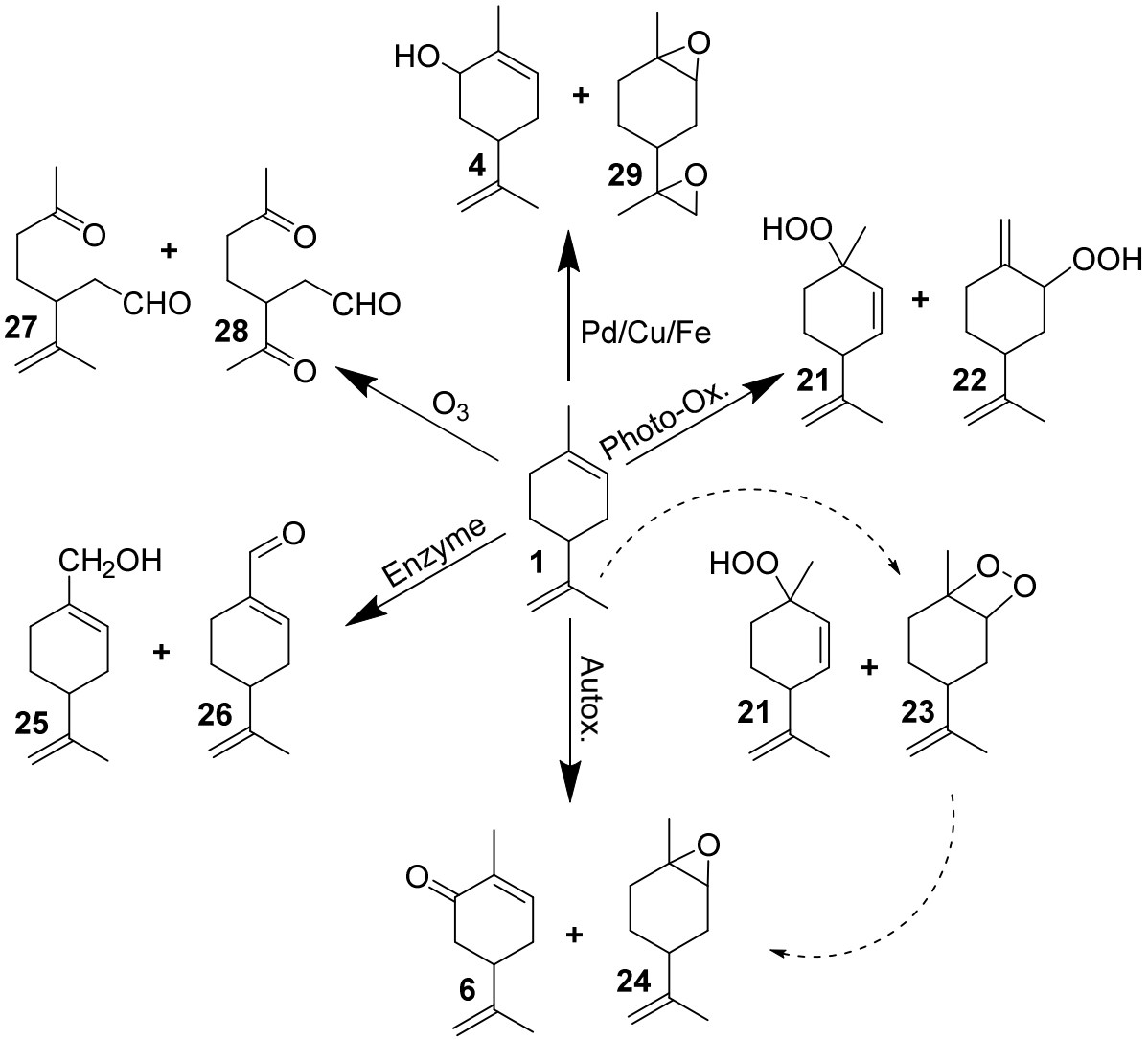

Figure 13. Illustration of different oxidation pathways of the terpene limonene (**1**) leading to various follow-up products. Major reaction products of different processes include carveol (**4**), carvone (**6**), limonene-1-hydroperoxide (**21**), limonene-2-hydroperoxide (**22**), limonene-dioxetane (**23**), limonene-1,2-epoxide (**24**), perillyl alcohol (**25**), perillaldehyde (**26**), limonene-aldehyde (**27**), keto-limonene-aldehyde (**28**), limonene-diepoxide (**29**).

3.6.1 Chemical changes in terpenoids in general

Plant terpenes are known to undergo chemical changes due to oxidation upon exposure to air or oxygen. Unsaturated compounds with one or more carbon double bonds (C=C) and allylic hydrogen atoms are prone to be attacked in oxidation reactions [138–140]. In autoxidation, a hydrogen atom is abstracted in an allylic position which results in the formation of a free radical. This process is immediately followed by the attachment of triplet oxygen ($^3O_2$) to the free radical and the formation of a hydroperoxide or dioxetane (Figures 13 and 14) [130]. Contrary to that, photo-oxidation is described by direct reaction of singlet oxygen ($^1O_2$) (which is formed from $^3O_2$ in this

process) with the terpene double bond. This reaction also leads to the formation of hydroperoxides [141]. Both reactions are enhanced by several factors such as light, heat, and catalysts such as Fe and Cu ions [28,130,141–143]. Consequently, isomerization, cyclization, dehydrogenation, and polymerization reactions may take place and result in new oxygen containing products [2,139]. In most cases, oxidation reactions also physically decrease the quality of essential oils by changes in odor, color, and viscosity [2,28]. However, the oxidation of fragrance compounds in essential oils not only impairs organoleptic properties. The resulting hydroperoxides are also considered to be potential sensitizing skin allergens [124,125,144]. The amount of hydroperoxides in the stored product is mainly dependent on the stability and amount of the individual terpenes and their primary oxidation products in the matrix [145]. Allergenic reactions have been observed especially in the context of limonene, linalool, linalyl acetate, and geraniol whereby different constitutional isomers and stereoisomers of the hydroperoxides have been described for the aforementioned substances [140,144,146,147]. Nevertheless, human susceptibility to skin-sensitizing fragrance compounds is strongly varying and should be individually checked [148,149]. Moreover, large differences exist in the allergenicity of hydroperoxide isomers formed from the same substance [144].

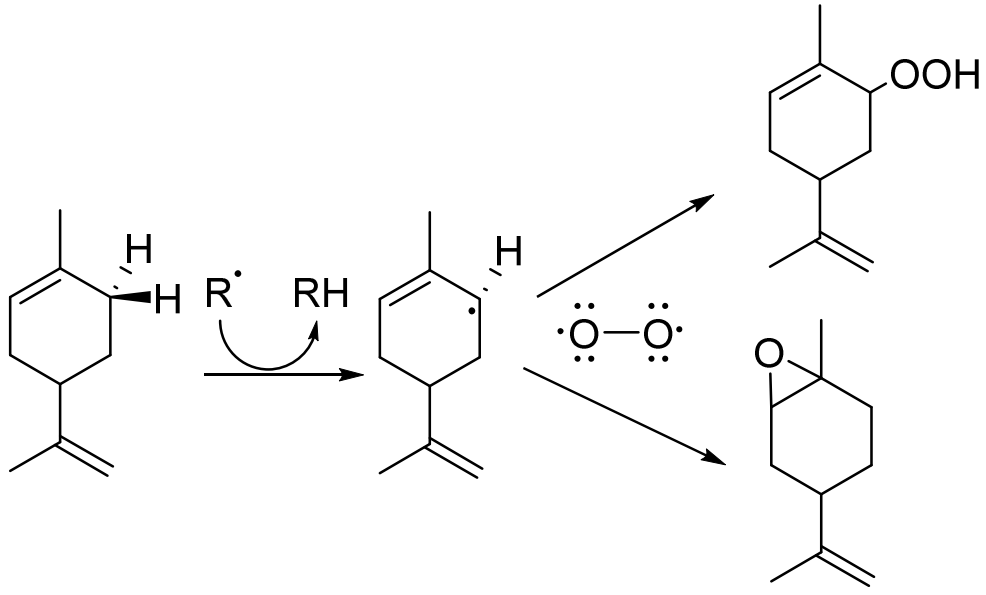

Figure 14. Illustration of the radical-catalyzed autoxidation process for limonene with triplet oxygen leading to a hydroperoxide (upper right) and epoxide (lower right).

3.6.2 The impact of oxygen

Contact with air or molecular oxygen is considered most decisive in the alteration processes of essential oils [2]. Oxygen available in the headspace of storage vessels is a crucial problem and its reaction is directly linked with the degree of terpene degradation [2,150]. Therefore, the stability of essential oils upon storage may be increased either by filling storage vessels up to the brim or by replacing air with noble gases such as argon [2,151]. However, also the amount of oxygen dissolved in the essential oil itself has an enormous influence on its stability [133]. The solubility is strongly dependent on the oxygen partial pressure above the oil as well as storage temperature [2]. Lower temperatures go along with a higher oxygen solubility and, also the accumulation of peroxyl radicals and hydroperoxides is preferred as they remain more stable in the cold [2,152]. Nevertheless, as shown by Nguyen *et al*. (2009) for lemon essential oil, considerable oxidative changes were also detected in oils during storage even under low air atmosphere or with inert gas. Apparently, the remaining oxygen dissolved in the essential oil was sufficient to initiate primary oxidation reactions [150]. Consequently, the introduction of oxygen from processing to storage should be minimized in any possible way.

3.6.3 The impact of light

In contrast to autoxidation, the term photo-oxidation describes an oxidative reaction mechanism which is initiated by the activation of triplet oxygen, thus leading to the excited singlet state ($^1O_2$) [28]. This activation to singlet oxygen is achieved by means of organic photosensitizers such as porphyrins, chlorophylls, or rose bengal [2,128,153]. Namely, the photosensitizers absorb light in the visible or UV-A range and get electronically excited. In a subsequent process, the bulk of the absorbed energy is transferred onto triplet oxygen to form the non-radicalic singlet molecule which bears a double bond. The highly electrophilic $^1O_2$ molecule can directly interact with double bonds of terpenoids in the so-called “ene” reaction which includes the abstraction of an allylic hydrogen atom to yield hydroperoxides as primary oxidation products [154]. By nature, this reaction occurs much faster than autoxidation [2,154]. Subsequently, the resulting hydroperoxides can also gradually react yielding secondary oxidation products such as esters, ketones, and alcohols as they have been described in autoxidation [2,139]. Photo-oxidation can be prevented by the absence of photosensitizers and shielding light by adequate storage vessels like brown glass or

aluminum [2]. However, photo-oxidation may be a problem of concern in end-user products since there is no control over storage anymore. Therefore, optimized packing material and advice on storage conditions should be provided since hydroperoxide formation should be strictly prevented.

3.6.4 The impact of temperature

According to the Arrhenius equation, $k = Ae^{\frac{-E}{RT}}$, whereby k is the reaction rate, A is the frequent factor, E is the activation energy, R is the universal gas constant, and T is the absolute temperature in Kelvin, temperature has a strong impact on the reaction rate of chemical reactions. The van't Hoff law derived therefrom roughly indicates a two-fold higher reaction rate when the temperature is increased by 10 K, respectively [2,22]. However, the solubility of oxygen in liquids decreases with increasing temperature (section 3.5.2) [155,156]. As demonstrated by Turek and Stintzing, essential oils respond differently to varying temperatures [2,152]. In rosemary and pine essential oils, storage stability and hydroperoxide formation was reduced at lower temperatures [152]. Contrary to that, lavender essential oil was strongly peroxidized if stored at 5 °C [2]. However, such investigations should also include pH and conductivity measurements because unstable hydroperoxides may already be degraded at higher temperatures and thus escape detection [2,152]. Similarly, different investigations on thermal essential oil stability indicated a substantial increase or decrease of characteristic marker compounds [150,157]. E.g., strong losses of γ-terpinene, β-caryophyllene, β-myrcene and β-pinene were accompanied with an increase of the amounts of *p*-cymene, *cis*- and *trans*-limonene oxide, *β*-caryophyllene oxide, and 1,8-cineole, respectively [150,158]. The investigations described in **paper 2** follow up on these results and showed comparable effects. As demonstrated by Yeh *et al*. (2013), the thermal stability of cinnamaldehyde could be strongly increased by the addition of eugenol, a well-known phenylpropanoid found in clove essential oil [159]. This example produced evidence that individual essential oil compounds may mutually influence each other (here: stabilization) upon thermal load [159].

3.6.5 Molecular structure characteristics

The oxidative stability of essential oil components is strongly dependent on their chemical structure. Unsaturated mono- and sesquiterpenes (sections 3.3.1 and 3.3.2) and especially those with conjugated double bonds may more easily form radicals and

are therefore prone to oxidation [2,28,150]. For instance, Turek and Stintzing showed that pine and turpentine oil which feature high shares of unsaturated terpenes such as α- and β-pinene aged faster than thyme essential oil [160]. Also, Neuenschwander *et al*. (2010) showed that olefinic double bonds readily react with peroxyl radicals to yield epoxides and alkoxyl radicals [142]. Likewise, autoxidation is predominantly initiated at allylic hydrogen atoms due to the formation of resonance-stabilized radicals after hydrogen abstraction [2,28]. As described before, in the “ene” reaction singlet oxygen can directly form hydroperoxides at carbon atoms with double bonds followed by a shift of the double bond as for instance in linalool or limonene [154,161,162].

3.6.6 Catalytic agents and reactive oxygen species

Trace impurities of metals such as copper (Cu^{+}/Cu^{2+}) and iron (Fe^{2+}/Fe^{3+}) can catalyze autoxidation reactions of essential oils [133,163]. These may enter the essential oils during distillation in classical copper stills or during storage in metal containers. Metal contaminants may force hydroperoxide decomposition and the formation of reactive oxygen species (ROS) such as alkoxy (RO•) or hydroxy (•OH) radicals. These radicals in turn can promote further steps in the reaction cascade of autoxidation. Moreover, iron contaminants may also be involved in the conversion of hydrogen peroxide with superoxide anions into singlet oxygen ($^1O_2$) followed by various reactions as shown above in the case of photo-oxidation [2,16,133]. Nevertheless, metal catalysts such as palladium have also been successfully used to generate new flavoring terpenoids [134]. In addition, enzymatic oxidations with high stereoselectivity have been described for several monoterpenes [164]. Also, ozone was shown to be a reactive ROS that can trigger oxidation and degradation of fragrance terpenes. This is of particular interest in environmental gas phase chemistry, since large amounts of terpenes such as α-pinene and β-pinene are released by coniferous trees into the atmosphere [165].

3.6.7 Modification and stabilization of essential oils

Relatively little is known about measures that can stabilize terpenes and essential oil components in final consumer products. For instance, encapsulation with stabilizers like gum arabic or modified starch may protect terpenes from oxidation [157,166]. The encapsulation technique comprises the coating of active (fragrance and flavor) material with different “shell” materials. The method is applied in pharmaceutical, chemical, cosmetics, and food industries. For volatile flavor and aroma components,

encapsulation is widely used in the food industry to reduce losses during processing and storage [167]. With agrumen essential oils being used in highest quantities (section 3.2), the food and beverage industry pursued different approaches for the stabilization of the key flavor compounds *R*-(+)-limonene and citral [166,168]. For instance, Djordjevic *et al*. (2007) and Charve *et al*. (2009) reported a remarkable increase in the stabilization of citral by using gum arabic possibly due to the protective matrix [166,168]. For limonene, laboratory experiments showed better results with soy protein isolate and sodium dodecyl sulfate [166,168]. Also, antioxidants (e.g., substituted resorcinols) have been used for terpenoid stabilization in cosmetic products [169]. Due to the rising demand for naturalness and sustainability by consumers, there is great interest in using plant compounds with protective properties [170,171]. As presented in **papers 3** and **4**, also furocoumarins may have a distinct protective effect on the light-induced oxidation of terpenes in essential oils.

3.7 Instrumental analysis of essential oils

Due to the complex composition of essential oils, adequate analytical techniques based on chromatographic separations are commonly applied for compound-specific analyses. The primary goal is the best possible separation of the individual compounds in the respective essential oil [172]. The chromatographic separation can be performed by GC (section 3.7.1), high-performance liquid chromatography (section 3.7.2), and thin layer chromatography (section 3.7.3), which are often coupled to mass spectrometry (MS) for structure investigation and verification [172]. By contrast, techniques without chromatographic separation such as titration (section 3.7.4), spectrophotometry (section 3.7.5), and NMR (section 3.7.6) are less frequently used in current days. Due to their use in the present work, the methods will be discussed in more detail in the following sections.

3.7.1 Gas chromatography (GC)

With certainty, gas chromatography is the most frequently used instrumental technique in essential oil analysis [172]. By their nature, essential oils are dominated by volatile terpenes with masses below 300 Da [59]. They are well suited for direct GC analysis since most of them can be volatilized without thermal degradation [173]. Today, separations are almost exclusively performed with capillary columns with lengths of up to 60 m and inner diameters of 0.25 or 0.32 mm. The inner walls of the capillaries are coated with thin films (e.g., 0.25 µm) of a polar (e.g., polyethylene glycol) or a non-polar (e.g., dimethyl polysiloxane) stationary phase [172,174]. Prior to analysis, essential oils are diluted in appropriate organic solvents, such as methyl-*tert*-butyl ether or they can be directly injected into the GC system. The analytes are carried by an inert gas flow (e.g. helium) through the separation column [173–175]. As an alternative, solvent-free samples can be analyzed utilizing head-space GC [175]. Hereby, aliquots of the equilibrated vapor above the sample containing the volatile compounds are used for analysis [173]. This can either be accomplished by direct sampling of a given volume of the gaseous space above the sample or by the combination of head-space sampling with solid phase microextraction (HS-SPME) [173,175]. In SPME, volatile compounds are first adsorbed on highly effective materials coated on small needles (Figure 15) [173,176].

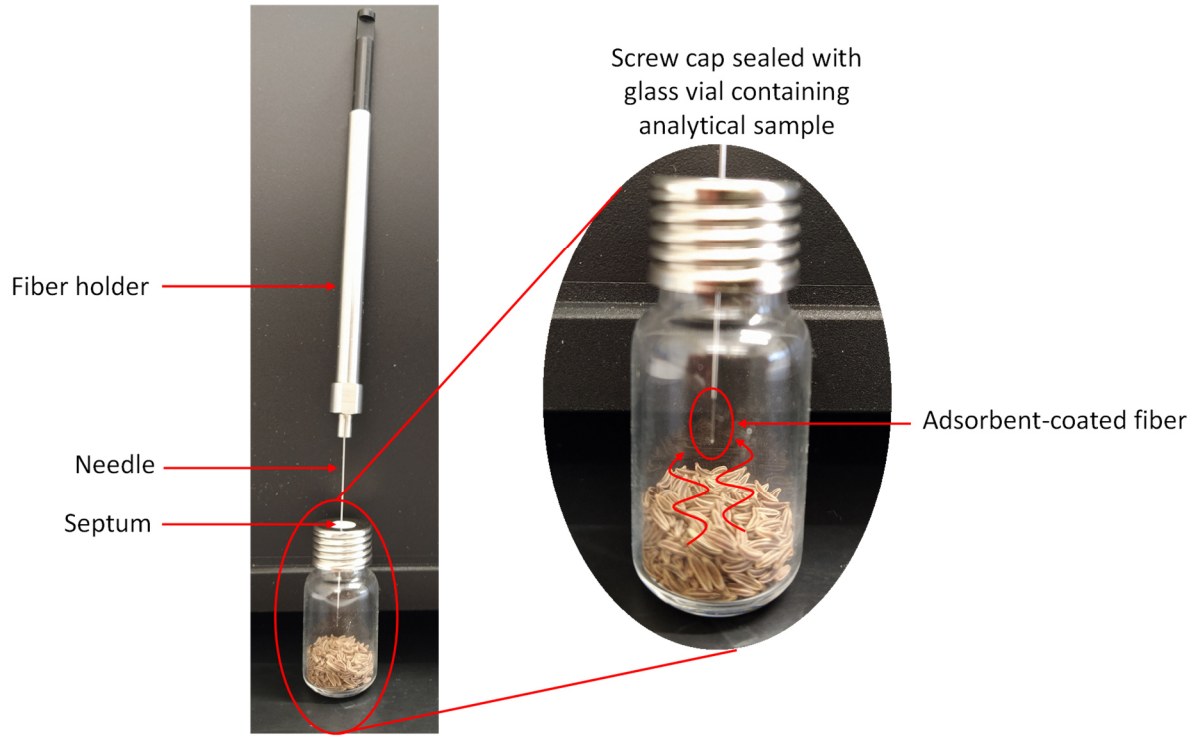

Figure 15. Illustrative representation of the HS-SPME analysis of flavor compounds from plant samples. Caraway seeds could be analyzed without previous essential oil distillation or extraction.

By the introduction of the loaded needle into the hot injector port of the GC, previously adsorbed volatiles are released from the sorbent again. Head-space GC does not involve solvents and omits the introduction of non-volatiles into the GC system the latter of which may contaminate sensible parts of the instrument [175,176]. Due to the concentration step of the volatiles on the SPME adsorption fiber, very low limits of detection (LOD) can be achieved with this technique [174–176]. The subsequent identification of the compounds after GC separation requires detectors that can accurately monitor all target substances and provide reproducible signals [172,174]. For qualitative analyses, the intensity of the signals should correlate with the amount of a substance [174]. The most frequently used GC detectors for essential oil analysis are the flame ionization detector (FID) and the mass spectrometer (MS) [1,177]. In an FID, the substances are combusted in an air/hydrogen flame and the resulting ions are captured and converted into a signal [178]. Hence, a signal in the GC/FID chromatogram at a given retention time indicates that at this moment, a substance had left the GC column and entered the FID.

Compared to that, the use of an MS detector not only indicates the elution of a compound from the column but additionally provides structural information which can

be helpful for the identification of unknown substances. GC-separated compounds are directly guided into the ion source in which they are bombarded with accelerated electrons in a vacuum (electron ionization, EI) [174,179]. Release of an electron from the intact molecule results in the positively charged molecular ion which is partly fragmented to give different positively charged fragment ions. These ions are subsequently separated according to their mass-to-charge (*m*/*z*) ratio [178,179]. Recorded with the same conditions, the resulting mass spectrum is characteristic of a given substance and, in comparison with available references and databases, the data can be used for rapid analyte identification (Figure 16) [174,178].

In **papers 1-4**, GC/MS was successfully used for the identification of essential oil compounds and degradation products formed in oxidation experiments. Moreover, in the case of flavoring compounds such as essential oils, the analysis of the odor (impression) and the individual impact of every single compound is of higher relevance [180]. In the so-called GC-olfactometry (GC-O) analysis the exit of the GC column is attached to a sniffing port where trained persons can sniff with their nose and record both the flavor note of a compound and the (GC retention) time when it is perceived [180,181]. This detection method is of great importance since the abundance of a peak in a GC/FID or GC/MS chromatogram (amount of substance) is not necessarily related to its contribution to the smell [180]. For instance, individual terpenes differ remarkably in their odor thresholds which is the lowest concentration at which they can be perceived by the nose [180–182]. The retention time of individual compounds is strongly affected by the GC conditions. For better comparability of results, internal standards are used which allow conversion to the retention times of the analytes into a dimensionless retention index system which was introduced by Kováts already in 1958 [172,174,183,184]. In **papers 1-4** RI values were additionally used to verify the identity of analytes.

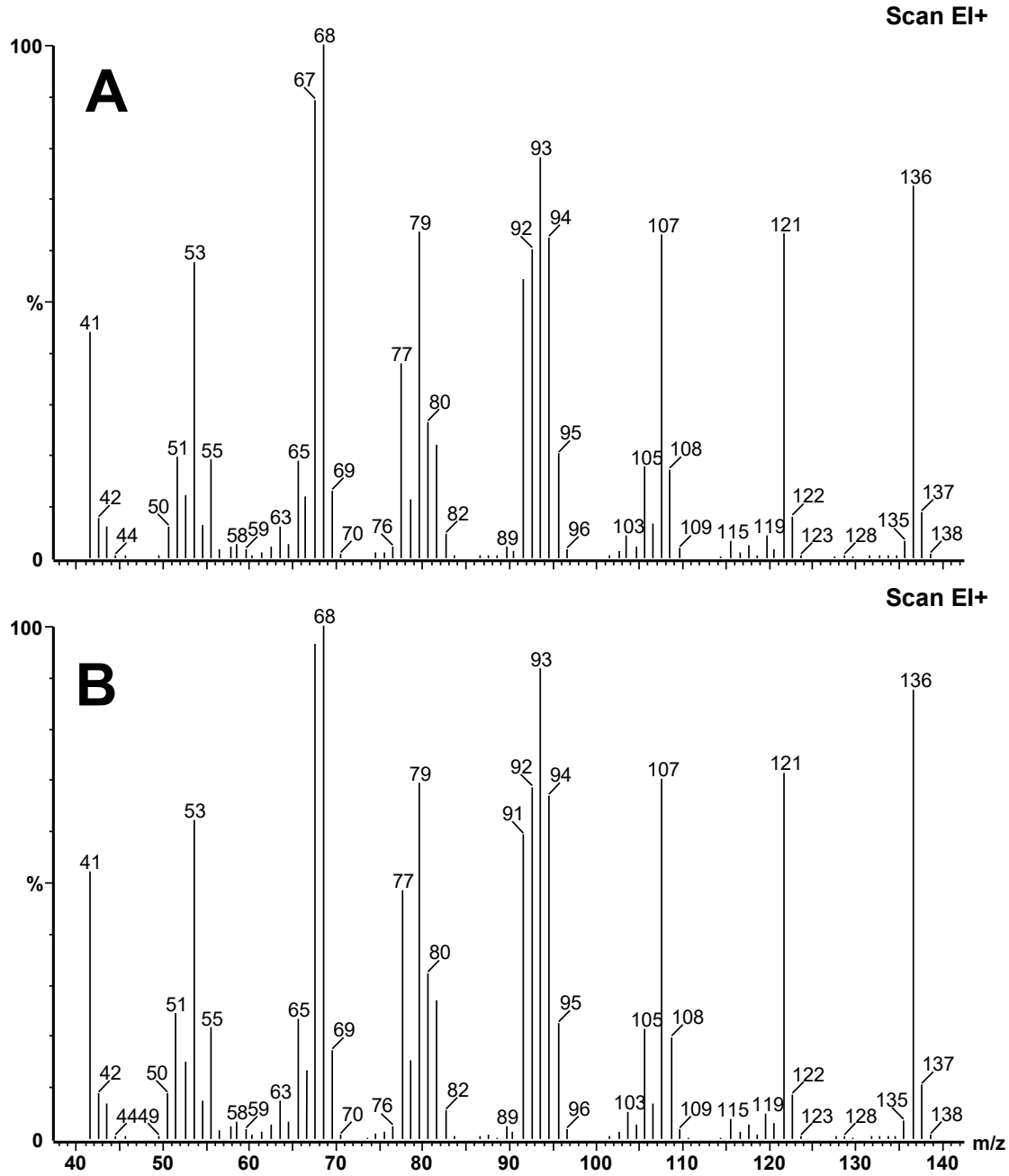

Figure 16. GC/MS spectra of *R*-(+)-limonene were obtained by measuring an authentic reference standard (**A**) and a lemon essential oil sample (**B**). The good match of GC retention time (not shown) and mass spectrum verifies the presence of the compound in the sample.

Further techniques which were not applied in the present thesis are for instance isotope ratio mass spectrometry (IRMS) whereby the stable isotopic composition is determined relative to a standard gas of known isotopic composition. Hereby more information about the geographical origin can be obtained [185]. Furthermore, enantioselective separations using homochiral cyclodextrin derivatives as (or as part of the) stationary phases have been used to determine the enantiomer ratio of chiral compounds. This

technique is often applied in the authenticity control of high-priced essential oils [58,186,187]. However, there are still major difficulties and challenges in the GC analysis of unstable and thermolabile compounds such as terpene hydroperoxides [188,189]. Some of these drawbacks can be overcome by the application of high-performance liquid chromatography (HPLC) which will be described in the following section.

3.7.2 High-performance liquid chromatography (HPLC)

First described in the 1970ies, essential oils have only scarcely been analyzed by HPLC given the simple applicability of GC in this research field (section 3.7.1). Nevertheless, some successful applications of HPLC have been described in the field of essential oils and terpenes [1]. The principal difference between HPLC and GC is the liquid nature of the mobile phase that is pumped with high pressure through the column densely packed with separating material. Contrary to GC, where the mobile phase is the same throughout the run, gradient elution can be performed in HPLC by changing the mobile phase composition to adopt the different polarities of individual analytes in a sample [172,178].

In HPLC, analytes are mostly detected by means of diode-array detectors (UV/Vis) which may additionally be coupled to a mass spectrometer [178]. Turek and Stintzing (2011) successfully investigated the composition of seven essential oils by HPLC. The simultaneous monitoring of the UV/Vis signal in a diode array detector (HPLC-DAD) and by mass spectrometry (HPLC-MS/MS) resulted in valuable structure information and characteristic terpenoid fingerprints, that may be used in quality control [190]. HPLC has also been repeatedly used in the analysis of cold-pressed essential agrumen oils [191,192]. As described by Russo *et al*. (2021), up to 15% of non-volatile substances located in the peel, including waxes, sterols, furocoumarins, and polymethoxyflavones were present in the essential oil [18]. These non-volatile essential oil compounds can be adequately assessed by HPLC [18,172]. Also, terpene hydroperoxides formed in the course of oxidative degradation (section 3.6.2), are difficult to analyze via GC because of their thermal instability [189]. Hence, following the pioneering work of Jones *et al*. (1980) who first described the first successful HPLC separation and identification of limonene hydroperoxides [193], this technique has been repeatedly used to detect compound alterations in essential oils as reported in **paper 2**. Indications of a possible role of terpene hydroperoxides in allergen responses

to fragrances which may lead to skin dermatitis also increased the interest in their analysis via HPLC, and progress has been made in recent years [189]. Sensitive HPLC-MS/MS methods are thus suited to overcome two intrinsic problems, i.e. (i) thermolability in GC and (ii) the lack of chromophores that limit the use of UV detectors [194].

However, especially the combined application of both methods – HPLC and GC – shall be suited to get a complete picture of the chemical processes. For instance, terpenoid analysis by GC can be accompanied by oxidation products by HPLC. This concept has been applied in **paper 2** for the analysis of oxidized caraway essential oils after storage over 18 months. Also, new HPLC approaches like multidimensional separation systems or enantioselective measurements have been applied in essential oil analysis. For example, the enantiomeric composition of the furanocoumarins in citrus oils was determined to show the botanical influence in various plants [195]. In addition, HPLC/MS was used to detect pesticide residues in essential oils [196].

3.7.3 Thin-layer chromatography (TLC)

Introduced in 1938, TLC is an analytical technique with a long tradition and is still being used today in the analysis of essential oils [197]. Although modern and (semi-) automated systems have been established (as described above), TLC can also be operated without expensive analytical equipment or sophisticated technical measuring instruments [1,178]. In TLC, the stationary phase is uniformly distributed as a thin layer on a glass or metal plate. When the lower part of the plate (~1 cm) is immersed into a suitable liquid phase, it will be transported up the plate by capillary forces. On its way, the mobile phase can move and separate the analytes dependent on their specific interaction with the stationary phase [178,198]. In this way, initial information can be gained about the substance classes present in a sample. In most cases, the adsorbent (stationary phase) consists of silica gel or aluminum oxide with specific chemical functional groups which can be combined with a fluorescence indicator [178].

After separation, a derivatizing step can be carried out with different agents after which compounds can be made visible as spots or bands on the plate [198]. The positions (heights) on the plate are referenced by means of the corresponding R_f value, which expresses the distance between the starting point and a spot or band on the plate relative to the front. The latter may be useful for compound verification by comparison

with reference standards [172,178]. A more advanced and powerful setup has been introduced by high-performance thin-layer chromatography (HPTLC). In HPTLC, semi-automated application systems are used in combination with plates of higher quality and the dosing of smaller volumes to improve the resolution of the analytes [178]. This enabled the successful application of HPTLC in essential agrumen oil authentication and the chromatographic fingerprinting of furocoumarins [199,200]. Also, in **paper 3** TLC was used to identify changes in the furocoumarin patterns during the photo experiments.

3.7.4 Titration

Analytical titration was first described by Claude Joseph Geoffray for acetic acid in vinegar, but there may be earlier references to this method [201]. This quantitative method is usually based on measuring the consumed volume of a specific standard solution after a complete reaction with the analytes [178]. In the case of essential oils, titration is mainly used for peroxide value determination by an iodometric redox reaction [202,203]. The peroxide value indicates the degree of aging of an essential oil via the content of hydroperoxides which are the primary oxidation products upon storage (section 3.6) [160,178]. The peroxide value can also be used to determine the shelf-life of a product. Moreover, indications for skin irritating effects caused by some monoterpenoid hydroperoxides add to the relevance of their determination in the quality control of essential oils [124,125,144,189]. Yet, quantitation of hydroperoxides by titration is difficult as they quickly form secondary products and thus escape detection [160,204]. Therefore, iodometric titrations of essential oils must always be critically evaluated whereby the previous history of the essential oil should also be taken into account. As described in **paper 1**, iodometric titration according to the European Pharmacopoeia was used to determine the hydroperoxide concentration of the reference standards used for the validation of the implemented spectrophotometric method.

3.7.5 Spectrophotometry (UV-Vis)

Spectrophotometric analytical methods are based on the absorption of electromagnetic radiation by the analytes upon excitation. For this measurement, the analyte is diluted, ideally with a non-absorbing solvent, and placed in a cuvette in the instrument. Then, light is passed through the cuvette, and it is measured which share of the light is

reaching the detector (i.e. the share that is not absorbed by the sample solution). The absorption strength depends on the layer thickness d (typically of a standardized cuvette) and the concentration of the substance c and is summarized in the Lambert-Beer equation $E = \varepsilon * c * d$ where E is the dimensionless extinction and ε is the compound-specific constant of proportionality [178]. Turek and Stintzing (2011) showed that most essential oil compounds have an absorption maximum below 200 nm since they are lacking double bonds and thus escape spectrophotometry. Nevertheless, HPLC-UV analysis at 220 nm allowed to determine the characteristic fingerprints of seven essential oils (section 3.7.2). Similarly, HPLC separation followed by mass spectrophotometric analyses prove to be useful in essential oil analysis as demonstrated by Turek and Stintzing (2011) [190].

In addition, a derivatization step prior to the spectrophotometric measurement can convert essential oil components into colored complexes (colorimetry) [178,205]. After reaction with 3,5-dinitrobenzoic acid, derivatized carvone, menthone, and pulegone could be successfully determined in caraway and dill essential oils [205]. Also, UV spectrophotometry was occasionally directly used in agrumen oil analysis. Determination of the strong UV-absorbing furanocoumarins enables a quick distinction between distilled and mechanically pressed essential oils [58]. In the present work, terpene-hydroperoxides could be successfully detected by spectrophotometry (**paper 1**). For this purpose, the analytes were derivatized with *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) dihydrochloride to yield the colored radical DMPD cations called Wurster's red.

3.7.6 Nuclear magnetic resonance (NMR)

Until now, essential oils as complete mixtures were rarely investigated by NMR [1]. ^{13}C NMR was used for the detection of individual compounds in essential oils. NMR analysis of authentic reference standards was used to create a spectral database with chemical shifts which enabled the subsequent analysis of essential oils. Tomi and co-workers (1995) were able to identify 92% and 95% of the terpenes present in juniper (*Juniperus communis*) and rosemary essential oils, respectively [206]. Furthermore, peaks co-eluting in GC could be unequivocally assigned by NMR. Also, non-volatile and thermolabile compounds which are difficult to analyze by GC could be determined with NMR spectroscopy [1,206]. In addition, ^{1}H NMR analysis enabled the detection of adulterations in essential oils of Citronella *Cymbopogon nardus* and *Cymbopogon*

winterianus [207]. Moreover, NMR analysis is still indispensable for the structure elucidation of individual components in a plethora of essential oil compounds [1]. The helpful structural information gained by NMR is also shown in **paper 1** for the elucidation of the limonene hydroperoxides. In addition, the identity and purity of several synthesized reference compounds could be determined by ^{1}H and ^{13}C NMR measurements (**paper 1-3**).

4 PUBLICATIONS

4.1 Paper 1

Rapid spectrophotometric method for assessing hydroperoxide formation from terpenes in essential oils upon oxidative conditions

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Rapid Spectrophotometric Method for Assessing Hydroperoxide Formation from Terpenes in Essential Oils upon Oxidative Conditions

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ABSTRACT: Essential oils are widely used in the food and cosmetics industry as natural flavoring and fragrance substances. For this reason, a thorough quality control applying selected analytical methods is required. Oxidation along with hydroperoxide formation is an important drawback during production and storage of essential oils. Hydroperoxides constitute the main products formed upon photo-oxidation of essential oils. Due to hydroperoxide instability, gas chromatography (GC) and high-performance liquid chromatography (HPLC) analyses are required. According to the European Pharmacopoeia, titration is the official method for oxidation assessment. However, this analysis is time-consuming, and large sample quantities are required. Here, we present a simple and accurate spectrophotometric method for the detection of peroxide trace amounts in essential oils and terpenes. The principle is based on the formation of Wurster's red, which is enforced by the peroxide-driven oxidation of *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD). The method was validated using dibenzoyl peroxide (DBP) and cumene hydroperoxide (CHP). To demonstrate the suitability of the method for routine analysis, various oxidized terpenes and essential oils were chosen. Moreover, photo- and thermal oxidation experiments were compared and evaluated using gas chromatography/mass spectrometry (GC/MS) and a synthesized limonene-2-hydroperoxide (Lim-2-OOH) reference standard to gather detailed information on the structural changes of the respective terpenes.

KEYWORDS: *photo-oxidation, thermal oxidation, rose bengal, UV/vis spectrophotometry, GC/MS*

INTRODUCTION

Essential oils are composed of volatile secondary plant constituents known for their pleasant smell and various biological activities, including antimicrobial, antiviral, anti-inflammatory, and antioxidant properties.[1−3] Dating back to the Egyptians, the medicinal use of essential oils from aromatic plants has not lost its meaning to this day.[1] Increasingly, they are used as natural fragrances in the food and cosmetics industry, in detergents and various consumer goods, with rising demand expected in the coming years.[1,4,5] The main components of essential oils are mono- and sesquiterpenes, including alcohols, esters, ketones, aldehydes, and epoxides.[1,6] Most essential oil components are hydrophobic and their molecular weight is below 300 Da, resulting in a low boiling point. Therefore, they can be easily isolated by steam- and hydrodistillation, even on an industrial scale.[1] Essential oil components are prone to chemical conversion during processing and storage, with oxidation playing a major role in this context.[6] A distinction can be made between autoxidation, a radical chain reaction, and photo-oxidation, where singlet oxygen reacts directly with double bonds of unsaturated molecules.[7,8] Heat, irradiation (UV light), and catalytically active agents, e.g., metal ions, are factors that enhance oxidation.[6] Photosensitizers such as chlorophylls or porphyrins play a key role as catalysts in photo-oxidation, which by nature is a much faster reaction than autoxidation. Both oxidation mechanisms lead to the formation of hydroperoxides as primary oxidation products.[6,7] The formation of hydroperoxides is a sign of aging, thus significantly affecting essential oil quality. Hydroperoxides may cause allergic skin reactions in sensitive human individuals, affecting 1−3% of the European population.[9−11] Thus, for consumer hazard appraisal, a clear identification of oxidized components is the basic prerequisite for quality control and consumer safety.[12] The analysis of essential oils is mainly performed by capillary gas chromatography. Because of the volatility of essential oil components and the additional information provided by mass spectrometry, this analysis is the method of choice.[13] However, the assessment of primary oxidation products is challenging due to hydroperoxide instability. Catalytically active surfaces and high temperatures in gas chromatography may destroy these compounds before detection. Previous derivatization or reduction into the

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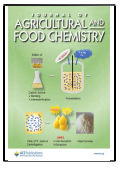

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corresponding alcohols is therefore necessary.[14−16] Alternatively, high-performance liquid chromatography (HPLC) methods have been described to circumvent these problems and to additionally obtain information of the composition due to previous chromatographic separation.[14,17] Currently, iodometric titration is the official method used to determine the peroxide content of essential oils.[18a,b] Decades ago, a spectrophotometric detection method for lauroyl and benzoyl peroxides was described by Levy and co-workers.[19] However, to the best of our knowledge, no spectrophotometric method for the detection of terpene hydroperoxides has been described so far. Therefore, the main objective of the present study was to develop a spectrophotometric method for the qualitative and quantitative determination of hydroperoxides from terpenes and essential oils. The fast and routine performance by the use of commercially available reagents, low detection limits, and simple analytical instruments is a major advantage compared to gas chromatography (GC), HPLC, and iodometry. In comparison to peroxide titration, the spectrophotometric method works on a microscale level and saves valuable and expensive raw materials.[18a,b] Moreover, photochemical and thermal oxidation experiments were carried out in model experiments with different terpenes and essential oils. To gather further information on reaction pathways upon oxidation, compound profiles were monitored by gas chromatography/mass spectrometry (GC/MS) experiments in parallel.

MATERIALS AND METHODS

Chemicals and Essential Oils. The following chemicals and reference compounds were used: *D*-(+)-limonene, *S*-(+)-carvone, and dibenzoyl peroxide (DBP) (Merck, Darmstadt, Germany); linalool, citral, linalyl acetate, *β*-citronellol, valencene, cumene hydroperoxide (CHP), *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), triethylamine (Et_3N), triphenylphosphine (PPh_3), and *m*-chloroperoxybenzoic acid (Sigma-Aldrich, Steinheim, Germany); rose bengal and (−)-carveol (Alfa Aesar-Thermo Fisher, Kandel, Germany); and (*Z*/*E*)-limonene-1,2-epoxide (Carl Roth, Karlsruhe, Germany). Na_2SO_4, NaCl, silica gel 60 (0.063−0.200 mm), and all solvents used were either of analytical or HPLC grade and purchased from Sigma-Aldrich, Merck, and Th. Geyer (Renningen, Germany). Diethyl ether (Et_2O) was distilled in a vacuum rotary evaporator (600 mbar, 38 °C) before use to remove the stabilization agent, butylated hydroxytoluene. Deionized water used for aqueous solutions and hydrodistillation was prepared with an Elga Purelab Classic Ultrapure Water System (Elga Labwater, Celle, Germany). Rose (*Rosa damascena* Mill.) and lemon (*Citrus limon* (L.) Osbeck) essential oils were obtained from Naturamus (Aichelberg, Germany).

Preparation of Caraway Essential Oil. Air-dried caraway (*Carum carvi* L.) seeds, grown in Bad Boll and obtained from the Sonnenhof farm (Bad Boll, Germany), were milled with a Baratza Virtuoso coffee grinder (Baratza, Bellevue, WA, USA) and sieved to a particle size ≤1000 *μ*m. Further, 200 g of ground seeds were immediately subjected to hydrodistillation using a 2 L three-neck round-bottom flask filled with 1100 mL of ultrapure water and equipped with a 30 cm distillation bridge. The system was heated with an IKA RCT standard heating plate (IKA, Staufen, Germany) at 180 °C and stirred at 260 rpm for 240 min using a KPG stirrer. The essential oil was separated from the aqueous phase using a separation funnel and subsequently stored under nitrogen at −85 °C for further analysis and oxidation experiments.

Photocatalytic Oxidation of Terpenes and Essential Oils. According to a previous procedure by Clark et al., a solution of terpene/essential oil (1.6 ± 0.1 g) and rose bengal (8.0 mg, 7.9 *μ*mol) in 40 mL of MeOH was stirred at room temperature under an O_2 atmosphere.[20] A 50 mL three-neck round-bottom flask equipped with an air balloon was filled with pure oxygen. Subsequently, the solution was illuminated with a Nano Power-LED (6500 K, 500 lm; Dennerle, Münchweiler an der Rodalb, Germany) and stirred at 600 rpm with an IKA RCT standard magnetic stirrer for 24 h. Afterward, MeOH was evaporated in vacuo and the reaction product was weighed. Then, the residue was subjected to silica column chromatography (ID 25 mm; 30 g of silica gel) to remove rose bengal. An *n*-hexane/Et_2O gradient was applied starting with 100% *n*-hexane and ending with a concentration of 20% (v/v) in Et_2O. The eluted fraction, devoid of rose bengal, was dried over Na_2SO_4, subsequently evaporated to dryness, and stored in 1.5 mL vials at −85 °C for further investigations. For all samples, experiments were carried out in duplicate. In addition, limonene (**1**) was subjected to thermal oxidation to simulate an aging process under light exclusion: 10.0 g of **1** was boiled under reflux for 6 h at 80 °C. The reflux condenser was attached to a three-neck 50 mL round-bottom flask equipped with a thermometer. The system was flushed and filled with O_2 using a balloon attached to the apparatus as applied for photo-oxidation.

Synthesis of Diastereomeric Limonene-2-hydroperoxide (*trans*-6, *cis*-7) and Limonene-Diepoxide. Limonene-2-hydroperoxide (Lim-2-OOH, **6**, **7**) was synthesized according to the procedure by Kao et al.[21] Briefly, 6.0 g of (−)-carveol (97%, 38.2 mmol) was added to an aqueous solution of hydrogen peroxide (50%, 85 mL) at 0 °C, acidified by five drops of conc. H_2SO_4.[21] The reaction apparatus was placed in a Dewar vessel and cooled by a mixture of ice/NaCl and vigorously stirred for 5 days at −18 °C. Subsequently, the reaction was quenched by adding 100 mL of deionized H_2O and extracted with 5 × 100 mL *n*-pentane. The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo. Then, 4.0 g of the crude product was purified by repeated column chromatography on silica gel, deactivated before rinsing with 0.01% (v/v) Et_3N in *n*-hexane. Gradient elution was started with 10% ethyl acetate (EtOAc) (v/v) in *n*-hexane increasing to a final concentration of 40% (v/v) EtOAc in *n*-hexane. The fractions obtained were analyzed by GC (92% *trans*-**6**), combined, and stored at −85 °C after evaporation in vacuo (542 mg, 8.4% of the theory). In addition, a limonene-diepoxide diastereomeric mixture was synthesized according to Gattermann and Wieland.[22] For this purpose, 2.0 g (14.7 mmol) of limonene (**1**) was treated dropwise with an excess of 8.6 g (49.8 mmol) of *m*-chloroperoxybenzoic acid dissolved in 110 mL of CH_2Cl_2. The reaction mixture was cooled to 0 °C and stirred for 2 h at 250 rpm. Afterward, a white precipitate was filtered off and washed with 2 × 50 mL of Na_2SO_3 solution (10%), 2 × 50 mL of saturated $NaHCO_3$ solution, followed by 2 × 50 mL of deionized H_2O. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. GC/MS analysis revealed three different limonene-diepoxide isomers of undefined conformation.

Reduction of Hydroperoxides into Alcohols and GC Analysis. Briefly, 262 mg (1 mmol) of PPh_3 was dissolved in 5 mL of *n*-hexane heated to 40 °C. The solution was added dropwise to 168 mg (1 mmol) of oxidized limonene. After precipitation of the PPh_3-oxide formed, the reaction mixture was cooled to −20 °C, and the supernatant was separated from sediments. The solution was directly filtered (2 *μ*m, poly(tetrafluoroethylene) (PTFE)) into a glass vial for subsequent GC/MS analysis. The same procedure was followed using CHP.

Analytical Characterization. *Spectrophotometric Analysis (UV/Vis).* A reagent solution was prepared according to a literature method described by Dugan.[23] Briefly, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (209.1 mg, 10 mmol) dissolved in 10 mL of deionized H_2O was transferred to an amber volumetric flask and made up to 100 mL with methanol. The solution was stored at 4 °C until analysis and prepared freshly for daily investigation. Prior to analysis, samples were dissolved in EtOAc in appropriate concentrations (0.01−5.0 mg/mL) and 1 mL of this sample solution was mixed with 1 mL of the reagent solution in 10 mL glass tubes, which were sealed with white plastic screw caps. Prior to this procedure, each glass tube was prewashed with acetone. After dishwashing and rinsing with deionized water, each tube was heated out for 2 h at 250 °C to remove any trace contaminants such as reducing organic compounds.

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Sample preparation was carried out in dimmed light because the reaction is sensitive to light.[19] Afterward, samples were incubated for 15 min at 50 °C prior to spectroscopic analysis using 1.5 mL poly(methyl methacrylate) (PMMA) semi-microcuvettes (Brand, Wertheim, Germany). A Lambda 2 UV/vis double beam spectrophotometer (Perkin Elmer, Waltham, MA, USA) was used for analysis. Full scan spectra were recorded from 900 to 300 nm, with a bandwidth of 2 nm. The spectrophotometric method was validated according to ICH Q2(R1) guidelines "Validation of Analytical Procedures: Text and Methodology" using VALIDAT 5.62.1691 software (iCD., Frechen, Germany).[24] The validation was performed using dibenzoyl peroxide (DBP), cumene hydroperoxide (CHP), and Lim-2-OOH obtained by synthesis (Synthesis of Diastereomeric Limonene-2-hydroperoxide (trans-6, cis-7) and Limonene-Diepoxide). Absorption was measured at λ_{max} 517 nm. Normal distribution was determined according to the Shapiro−Wilk test, and outliers were determined according to Dixon's Q test. The concentration of the hydroperoxides used for validation was determined by iodometric titration according to Ph. Eur. 9. basic volume 2017, using a semiautomated titration system Mettler Toledo T90 (Mettler Toledo, Columbus, OH, USA).

Gas Chromatography/Mass Spectrometry (GC/MS). GC/MS experiments were performed with a Perkin Elmer Clarus 500 gas chromatograph equipped with a split/splitless injector coupled to a Perkin Elmer Clarus 500 Mass Selective Detector with a quadrupole mass filter. Data acquisition and control of the system was achieved with TurboMass software, version 6.1.2 (Perkin Elmer). The injection volume was 1.0 μL with a split ratio of 1:20 for samples dissolved in methyl *tert*-butyl ether (MTBE). Helium was used as carrier gas at a constant flow of 1.0 mL/min. The analytical column was coated with 5% phenyl 95% poly(dimethylsiloxane) (60 m × 0.25 mm i.d., d_f = 0.25 μm, ZB-5ms, Phenomenex, Torrance, CA, USA).

The following temperature program was applied: initial temperature 60 °C; isothermal hold for 4 min at 60 °C; an increase to 240 °C (6 °C/min) followed by a ramp to 280 °C (8 °C/min); and then an 8 min isothermal hold. The total run time was 47 min. Source and transfer line temperatures were set to 180 and 220 °C, respectively. Mass spectra and total ion chromatograms were recorded in electron ionization (EI) mode covering a scan range of m/z 30−300. Data evaluation of the chromatograms (TIC and selected ion monitoring (SIM)) was performed with TurboMass Data Analysis Application (Perkin Elmer). The compound assignment was based on mass spectra, comparison of linear retention indices (LRIs) determined according to van den Dool and Kratz, with the NIST database, and using authentic reference standards if available.[25]

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra were recorded in $CDCl_3$ at 600 (^{1}H) and 150 MHz (^{13}C) using a 600 MHz Bruker Avance III HD NMR spectrometer. Chemical shifts are reported in δ [ppm] and refer to residual solvent signals of $CDCl_3$ (^{1}H: 7.27; ^{13}C: 77.00 ppm). NMR spectra were evaluated by means of the SpinWorks 3.1.7 software (Copyright 2010, K. Marat, University of Manitoba, Winnipeg, MB, Canada).

RESULTS AND DISCUSSION

The following section describes the development and establishment of the spectrophotometric method using DMPD reagent to monitor hydroperoxide formation. Accelerated oxidation experiments using catalytic rose bengal, acting as the singlet oxygen generator (photo-oxidation) in comparison to air oxidation at elevated temperatures (thermal oxidation), were carried out.[20] The oxidation products obtained from different terpenes and essential oils were evaluated by means of spectrophotometry. In addition, the analysis of the oxidation products was performed using GC/MS to obtain further information on primary hydroperoxide formation and consecutive reaction pathways.

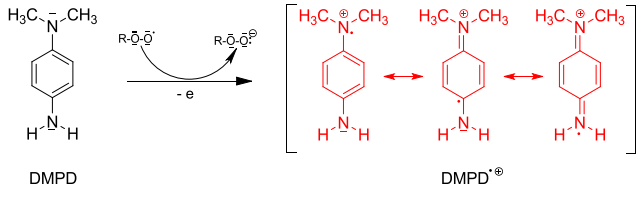

Figure 1. Oxidation of DMPD by a hydroperoxy radical to red-colored resonance-stabilized radical DMPD cations (Wurster's red). Illustration modified according to ref 27.

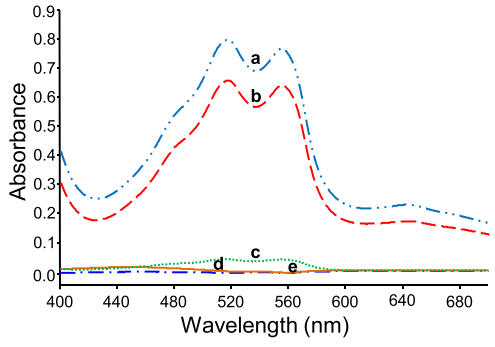

Figure 2. UV/vis spectrum of photo-oxidized limonene (a), cumene hydroperoxide (b), limonene reference standard prior to oxidation (c), and both PPh_3-reduced photo-oxidized limonene (d) and cymene hydroperoxide (e). All samples after reaction with DMPD reagent.

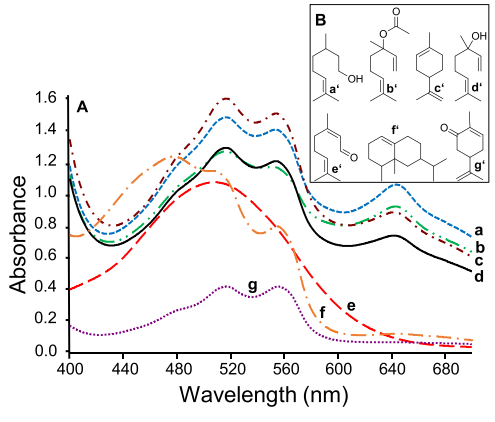

Figure 3. (A) Spectrophotometric analysis of photo-oxidized β-citronellol (a) 1 mg/mL, linalyl acetate (b) 1 mg/mL, limonene (c) 1 mg/mL, linalool (d) 1 mg/mL, citral (e) 0.01 mg/mL, valencene (f) 1 mg/mL, and carvone (g) 2 mg/mL. All samples after reaction with DMPD reagent. (B) Structure of starting compounds (a′−g′) prior to photo-oxidation.

Spectrophotometric Analysis of Hydroperoxides and Method Validation. The reaction mechanism allowing peroxide detection with *N*,*N*-diethyl-*p*-phenylenediamine has been described by Bader et al. as a radical mechanism.[26] Disubstituted *p*-phenylenediamines (DMPDs) were oxidized to resonance-stabilized radical cations with a fairly stable red color called Wurster's red.[26] Accordingly, low concentrations of hydroperoxides can be detected because of the high specific absorbance of the radicals at 517 and 545 nm.[26−28] The oxidation of DMPD by means of electron subtraction due to a hydroperoxy radical leads to a resonance-stabilized DMPD radical cation, as illustrated in Figure 1. Based on this principle, the spectrophotometric method was validated as described in the Spectrophotometric Analysis (UV/Vis) section. The linearity of the system was determined by a six-point

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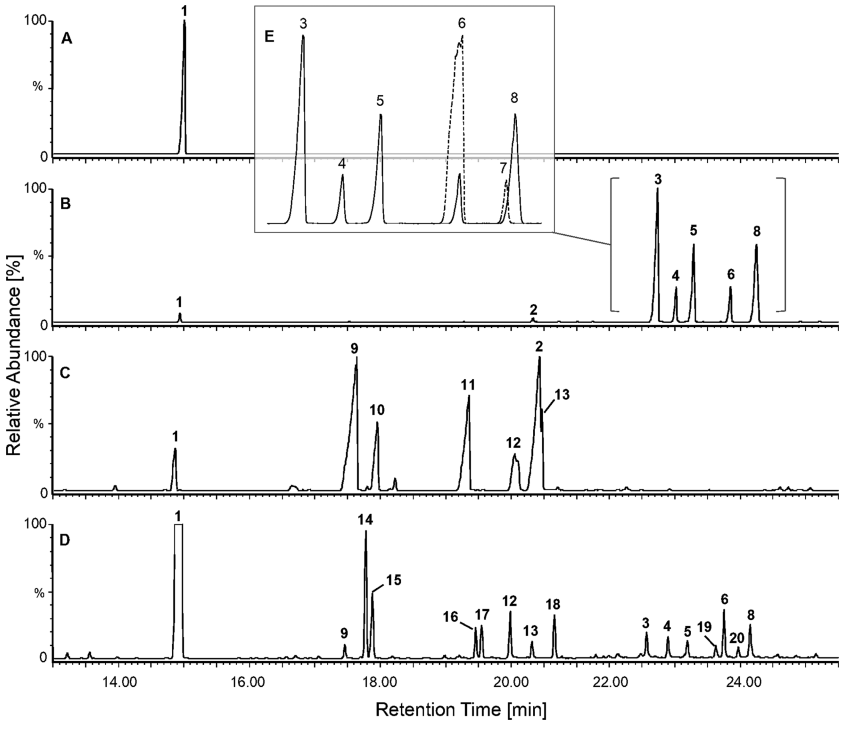

Figure 4. TIC EI + chromatograms of limonene (A), photo-oxidized limonene (B), and PPh3-reduced photo-oxidized limonene (C). (D) illustrates a chromatogram of limonene after thermal oxidation. (E) shows an overlay of the photo-oxidized limonene (-) and the synthesized reference compound (--). For peak assignment see Table 1.

calibration graph (50−800 ppm) resulting in a correlation factor of 0.998, 0.982, and 0.974 for DBP, CHP, and Lim-2-OOH, respectively. The system precision was investigated by a six-time repeated analysis of the same sample. Following this procedure, the relative standard deviation for the three model compounds was 0.14% (DBP), 1.36% (CHP), and 2.75% (Lim-2-OOH). All substances showed a normal distribution of the data ($p < 0.05$, Shapiro−Wilk test). Repeatability was determined through analysis by two different persons. Herein, the relative difference for DBP was 0.62 and 0.96% for CHP, also showing normal distribution and no outliers according to Dixon's test. The limit of detection (LOD) and limit of quantitation (LOQ) were determined as follows: LOD = 3.3 σ/S and LOQ = 10 σ/S, whereby σ defines the standard deviation of the blank and S is the gradient of the calibration curve. LOD (LOQ) was 0.5 ppm (1.5 ppm) for DBP and 2.0 ppm (6.2 ppm) for CHP. With a detection limit of 0.3 μg/L, Bader et al. reported even lower values for the detection of H_2O_2 in water using *N*,*N*-diethyl-*p*-phenylenediamine.[26] It should be mentioned that higher LODs have been described for hydroperoxide assessment by GC and also that prior derivatization is required to yield the corresponding trimethylsilyl derivatives.[15,29] The spectrophotometric results of the present investigation yielded similarly low LODs for photo-oxidized linalool (<5 ppm), linalyl acetate (<5 ppm), and limonene-2-hydroperoxide (<5 ppm) as earlier reported by LC/MS analysis.[30] The recovery of the photometric analysis was determined by spiking experiments. Standard amounts of CHP and DBP (75, 100, and 125%) were added to pure limonene (**1**) and EtOAc, respectively. For the three test concentrations, accuracy with DBP (96; 101; 95%) was slightly better than with CHP (88; 104; 126%). Stronger deviations from the target value obtained with the hydroperoxide CHP may be due to its greater instability compared to the peroxide DBP. This finding was also earlier reported using *tert*-butyl hydroperoxide.[31] To prove that Wurster's red formation is only due to hydroperoxides formed, oxidized samples and CHP were reduced with PPh_3 as the negative control. Afterward, no characteristic absorption was observed, as illustrated in Figure 2.

Analysis of Oxidized Terpenes. In total, seven different terpenes were analyzed by spectrophotometry. To prove that distinct functional groups in terpenes do not interfere with the analytical method, different terpene substrates were analyzed by DMPD reagent before oxidation. None of the substances led to Wurster's red formation prior to photo-oxidation. Spectrophotometric analysis of six photo-oxidized terpenes after reaction with DMPD (Figure 3) resulted in the highest absorbance with limonene (**1**), known for the rapid formation of hydroperoxides.[16] Five hydroperoxide isomers of limonene were detected by GC/MS as shown in Figure 4B and Table 1 (compounds **3**−**6** and **8**). Likewise, formation of hydroperoxides from β-citronellol, linalool, and linalyl acetate could be verified by spectrophotometry (Figure 3A, spectra a, b, and d). Moreover, an almost complete conversion of the original terpenes was detected by GC/MS. The formation of two main reaction products (hydroperoxides) upon photo-oxidation of β-citronellol, linalool, and linalyl acetate was in agreement with literature reports.[32−34] However, oxidized citral (Figure 3A, spectrum e) did not show the diagnostic absorption bands of Wurster's red at 517 and 545 nm, respectively. This could be an indication that stable hydroperoxides were not formed and interference with other oxidation products took place.[7] Valencene showed additional absorption at 470 nm, which possibly was triggered by self-absorption and polymeric structures of the bicyclic sesquiterpene. Finally, the lowest peroxide content was determined upon oxidation of *S*-(+)-carvone (**18**). This finding was corroborated by GC

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Table 1. Compounds Formed upon Photo- and Thermal Oxidation of Limonene (1) and Subsequent Reduction of Hydroperoxides Identified by GC/MS as Illustrated in Figures 4 and 8

no.	compound	RI_a	RI_b	identification	refs
1	(4*R*)-limonene	1030	1030	RI, GC/MS[a]	40
2	(2*R*,4*R*)-*p*-mentha-[1(7),8]-diene-2-ol	1228	1235	RI, GC/MS	41
3	(1*S*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide	1308	1312	RI, GC/MS	16
4	(1*R*,4*R*)-*p*-mentha-2,8-diene-1-hydroperoxide	1321	1324	RI, GC/MS	16
5	(2*S*,4*R*)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide	1332	1338	RI, GC/MS	16
6	(2*S*,4*R*)-*p*-mentha-6,8-diene-2-hydroperoxide (*trans*-limonene-2-hydroperoxide)	1357	1365	RI, GC/MS[a]	16
7	(2*R*,4*R*)-*p*-mentha-6,8-diene-2-hydroperoxide (*cis*-limonene-2-hydroperoxide)	1372	1381	RI, GC/MS[a]	16
8	(2*R*,4*R*)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide	1375	1381	RI, GC/MS	16
9	(1*S*,4*R*)-*p*-mentha-2,8-diene-1-ol	1124	1123	RI, GC/MS	42
10	(1*R*,4*R*)-*p*-mentha-2,8-diene-1-ol	1135	1138	RI, GC/MS	42
11	(2*S*,4*R*)-*p*-mentha-[1(7),8]-diene-2-ol	1189	1185	RI, GC/MS	43
12	(2*S*,4*R*)-*p*-mentha-6,8-diene-2-ol (*trans*-carveol)	1217	1216	RI, GC/MS[a]	40
13	(2*R*,4*R*)-*p*-mentha-6,8-diene-2-ol (*cis*-carveol)	1234	1226	RI, GC/MS[a]	40
14	*cis*-limonene-1,2-epoxide	1129	1131	RI, GC/MS[a]	40
15	*trans*-limonene-1,2-epoxide	1133	1141	RI, GC/MS[a]	40
16	isomer of *p*-mentha-dieneol	1193		GC/MS[b]	
17	isomer of *p*-mentha-dieneol	1197		GC/MS[b]	
18	(4*S*)-carvone	1242	1240	RI, GC/MS[a]	40
19	isomer of limonene-1,2:8,9-diepoxide	1347		GC/MS[a,b]	
20	isomer of limonene-1,2:8,9-diepoxide	1363		GC/MS[a,b]	

[a]RI_a, retention indices relative to C_8−C_{20} *n*-alkanes; RI_b, reference indices; identified by comparison with the reference standard. [b]Tentatively identified.

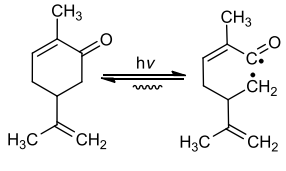

Figure 5. Proposed Norish-Type-I reaction scheme for carvone characterized by homolytic photochemical cleavage in α-position and subsequent recombination to the initial structure.

analysis showing only minimal compound degradation. The stability of carvone may be explained by a Norish-Type-I photoreaction known from ketones and aldehydes, which is induced by homolytic cleavage of the α-carbon bond (Figure 5). Subsequent combination of the two radicals obtained results in the reformation of the analytes.[35] In contrast to photo-oxidation, thermally oxidized limonene (**1**) showed a lower absorption by Wurster's red formation, indicating lower amounts of hydroperoxides. *Cis*- and *trans*-limonene-1,2-epoxides (**14**, **15**) were the main products from thermal

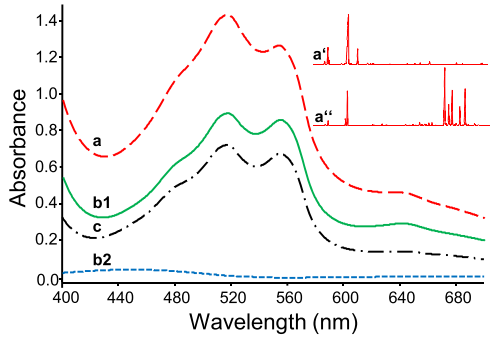

Figure 6. Spectrophotometric analysis of photo-oxidized lemon (a) 1 mg/mL, caraway (b1) 1 mg/mL, and rose essential oil (c) 1 mg/mL. Graph (b2) shows a nonoxidized fresh caraway essential oil (1 mg/mL) distilled as described in the Preparation of Caraway Essential Oil section. All samples analyzed after reaction with DMPD reagent. GC/MS EI + chromatogram of nonoxidized lemon oil (a′) and photo-oxidized lemon oil (a″).

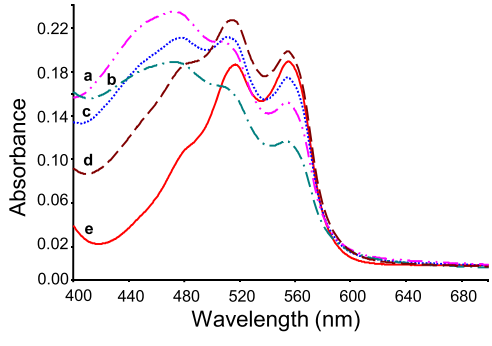

Figure 7. UV/vis spectra of caraway essential oil samples stored under unknown conditions since 2009 (a) 5 mg/mL, 2005 (b) 5 mg/mL, 2013 (c) 5 mg/mL, 2003 (d) 5 mg/mL, and cumene hydroperoxide reference standard (e) 100 ppm. All samples after reaction with DMPD reagent.

autoxidation that neither reacted with DMPD nor produced signals interfering with the spectrophotometric determination of hydroperoxides. However, both hydroperoxides and epoxides of limonene could be detected by GC/MS (Figure 4D). Similarly, both substance classes along with hydroxy and dihydroxy degradation products could be detected in these experiments (Table 1, compounds **3**−**6**, **8**, **9**, **12**−**20**). Further photo-oxidation experiments of limonene without catalytic rose bengal also led to the preferential formation of hydroperoxides, but the reaction rate was much lower.

Analysis of Oxidized Essential Oils. In addition to single reference compounds, essential oils, which represent multi-compound blends, were analyzed on oxidation products by spectrophotometry. All tested fresh essential oils did not reveal hydroperoxides prior to oxidation experiments. Upon oxidation, lemon oil was most sensitive to O_2 as deduced from the highest absorption in spectrophotometry (Figure 6). In agreement with this, the present lemon oil consisted of 76% limonene (**1**) and 9% β-pinene, both being prone to oxidation.[36] By contrast, caraway and rose oils showed a significantly lower absorbance under the same oxidation conditions, as illustrated in Figure 6. This may be due to the complex composition of essential oils: the high proportion of carvone (**18**) in caraway and the predominance of linear alkanes such as heptadecane, nonadecane, and heneicosane in rose oils may explain the significantly lower peroxide

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Table 2. ^{1}H and ^{13}C NMR Data of *trans*- (6) and *cis*-Limonene-2-hydroperoxide (7) in $CDCl_3$ (δ in ppm)

compound	6			7		
position	δ_c[a]	δ_H (mult., *J* in Hz)[b]	HMBC	δ_c[a]	δ_H (mult., *J* in Hz)[b]	HMBC
1	82.54	4.36 (dd, *J* 3.1, 2.1 Hz)	C2, C3, C5, C7	84.25	4.56–4.51 (m)	n.o.[c]
2	129.29			132.79		
3	129.70	5.75 (br d, *J* 5.9 Hz)	C1, C4, C5, C7	127.08	5.66–5.63 (m)	C1, C4, C5, C7
4	31.11	2.16 (br dt, *J* 17.4, 5.4 Hz); 83 (br dd, *J* 17.5, 12.0 Hz)[e]	C2, C3, C5, C6; C2, C3, C5	30.86	2.07 (br d, *J* 17.5 Hz); 1.99–1.91 (m)	C2, C3, C5, C6[d]
5	35.15	2.37 (br tt-like, *J* 12.8, 3.9 Hz)[e]	C1, C2, C4, C8, C9, C10	40.50	2.28 (br ddt, *J* 12.0, 4.5, 2.7 Hz)	C1, C4, C8
6	31.16	2.37 (br ddd, *J* 14.9, 2.6, 3.4 Hz)[e]	C1, C2, C4, C5, C8; C4, C5	32.47	2.21 (ddt, J 12.1, 5.9, 2.2 Hz)[f]	C1, C2, C4, C5, C8[d]
		axial: 1.46 (ddd, *J* 14.2, 13.3, 3.5 Hz)			axial: 1.79 (ddd, *J* 10.0, 11.8, 12.4 Hz)[f]	
7	21.20	1.80 (s)	C1, C2, C3	19.12	1.80 (s)[d]	[d]
8	149.17			148.70		
9	109.03	4.78 (br s); 4.77 (br s)	C5, C8, C10	109.23	4.77 (br s)[f]	C5
10	20.88	1.75 (s)	C5, C8, C9	20.46	1.74 (s)	C5
11 (OOH)		7.98 (br s)	C1		7.63 (br s)	n.d.

[a]Recorded at 150 MHz. [b]Recorded at 600 MHz; coupling constants were directly taken from spectra and are not averaged. [c]n.o.: Not observed due to low concentration of 7. [d]Overlapped signal. [e]Multiplicity and coupling constants derived from 1D projections of non-decoupled HSQC. [f]Multiplicity and coupling constants derived from selective 1D TOCSY.

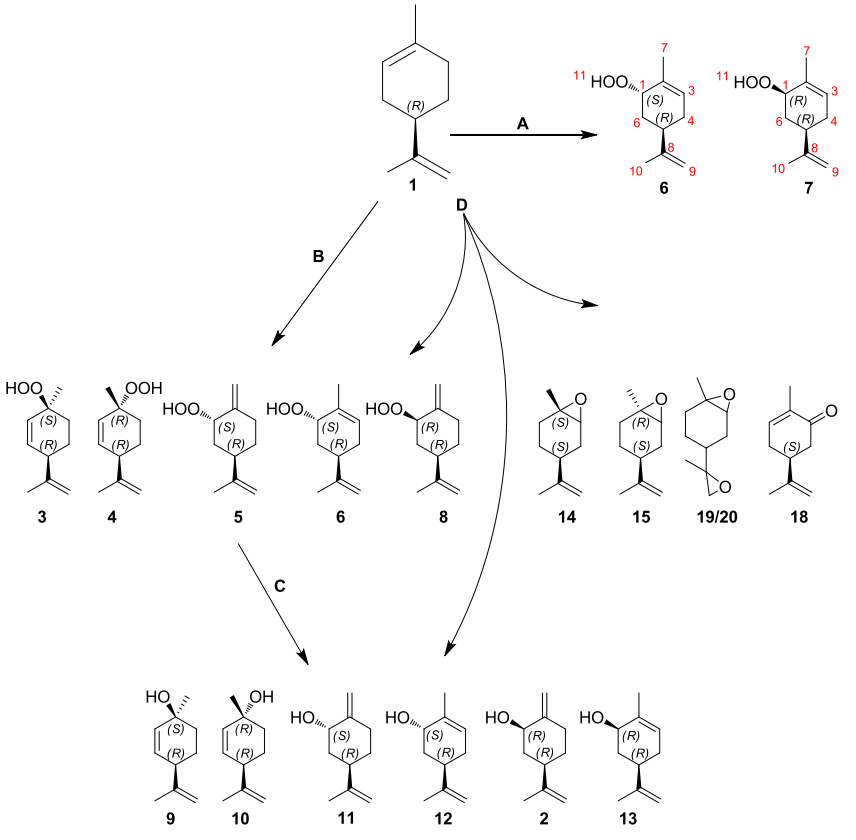

Figure 8. Pathways of limonene (**1**) transformation by reaction with H_2O_2 (A), photo-oxidation with rose bengal (B), reduction with PPh_3 (C), and thermal oxidation (D). Atom numbering of compounds **6** and **7** according to NMR structure elucidation.

values.[37,38] Since carvone itself is an oxidation product of limonene and linear alkanes in rose lack unsaturation, both oils are not sensitive to singlet oxygen attack. Finally, the applicability of the spectrophotometric method for routine purposes was tested by the analysis of commercial caraway essential oils after several years of storage (Figure 7). For semiquantitative determination of the peroxide content, one sample was spiked with 100 ppm CHP, which served as a reference. Based on the reference sample, a peroxide content below 2% was determined in the essential oils. As a result, caraway oil with carvone as the main compound appears relatively stable toward peroxide formation for years. However, the same aged essential oils showed a yellow color, along with an additional absorption between 400 and 500 nm, as displayed in Figure 7.

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Table 3. Gravimetric Changes of Terpenes and Essential Oils upon Photo-Oxidation with Rose Bengal

sample	molecular weight ($M_{terpene}$) (g/mol)	initial weight (W_{t_0}) (g)	weight measured (W_m) (g)	percentage weight gain (%)	molecular weight as R−OOH (M_{R-OOH}) (g/mol)	theoretical weight as R−OOH ($W_{t\ R-OOH}$) (g)	oxidation factor $\delta = W_m/W_{t_{R-OOH}}$
limonene	136.234	1.611	1.843	14.4	168.234	1.989	0.93 ± 0.04
carvone	150.218	1.618	1.655	2.3	182.218	1.963	0.84 ± 0.02
linalool	154.249	1.608	2.017	25.5	186.249	1.941	1.04 ± 0.00
citral	152.233	1.616	2.241	38.7	184.233	1.955	1.15 ± 0.00
linalyl acetate	196.286	1.610	1.885	17.1	228.286	1.872	1.01 ± 0.00
β-citronellol	156.265	1.611	1.986	23.3	188.265	1.940	1.02 ± 0.02
valencene	204.351	1.613	1.704	5.6	236.351	1.865	0.91 ± 0.00
rose oil	199.244[a]	1.611	1.925	19.5	231.244	1.870	1.03 ± 0.00
lemon oil	136.392[a]	1.632	1.549	−0.4	168.392	2.015	0.81 ± 0.02
caraway oil	142.183[a]	1.608	1.641	2.1	174.183	1.970	0.83 ± 0.01

[a]Average value calculated based on relative composition as deduced from GC/MS analysis. Weight measured (W_m) and theoretical weight ($W_{t_{R-OOH}}$) values are shown as mean values; owing to different initial weights, no standard deviation is possible. ± = standard deviation. All experiments and analyses were performed in duplicate.

Comparison of GC/MS Profiles of Limonene after Photo-Oxidation and Thermal Oxidation. To assess a broad spectrum of potential oxidation products, two different experimental approaches were compared mimicking photo- and thermal oxidation (Photocatalytic Oxidation of Terpenes and Essential Oils) of limonene (**1**). Five different limonene hydroperoxides (Table 1; **3**, **4**, **5**, **6**, and **8**) were identified by GC/MS analysis as photo-oxidation products by alignment with the NIST database and retention indices relative to C_8−C_{20} *n*-alkanes.[16] For comparison, Lim-2-OOH (**6**, **7**) was additionally synthesized as reference material according to a literature procedure by reaction of (-)-carveol with 50% H_2O_2.[21] After purification by repeated column chromatography, a mixture of *trans*-/*cis*-Lim-2-OOH diastereomers (**6**, 92%; **7**, 8%) was obtained (yield: 8.4% of the theory). Structure assignment of the diastereomers **6** and **7** was based on one-dimensional (1D) and two-dimensional (2D) NMR data (^{1}H, ^{13}C, HMBC, and gHSQC) and comparison with literature data (Table 2).[17,21] However, advanced and more specific signal assignments were carried out by selective 1D TOCSY experiments (Table 2). Photo-oxidation of limonene (**1**) with rose bengal preferably yielded (1*S*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide (compound **3**, 41%, Table 1), as illustrated in Figure 4B.[16] Limonene hydroperoxides **4** (7%), **5** (19%), and **8** (26%) were also clearly identified by GC/MS analysis. For compounds **5**, **6** (7%), **7**, and **8**, we found a different *R*/*S* stereochemistry than described earlier.[16] Figures 4C and 8 show the corresponding pairs of diastereomeric alcohols **2**/**11**, **9**/**10**, and **12**/**13** formed from the hydroperoxides **8**/**5**, **3**/**4**, and **6**/**7**, respectively, after reduction with PPh_3. While hydroperoxides **7** and **8** resulting from limonene photo-oxidation were not resolved by GC, the presence of both diastereomers could be verified after their reduction by means of the corresponding alcohols **2** and **13** in GC analysis (Figures 4B,C and 8). In comparison, thermal oxidation of limonene (**1**) resulted in a variety of different reaction products, and **1** was less affected by heat than by light. Limonene hydroperoxides (**3**, **4**, **5**, **6**, and **8**) appeared in low concentrations, whereas a pronounced epoxide (**14**, **15**, **19**, **20**) formation was observed. *Cis*- and *trans*-limonene-1,2-epoxides were the main products (**14** and **15**, Figure 4D). Epoxides have earlier been described as thermal oxidation products of terpenes including **1**.[39] Compounds **19** and **20** (limonene-1,2:8,9-diepoxides, Figure 8) showed mass spectra similar to that of a synthesis product obtained by reaction of **1** with *m*-chloroperoxybenzoic acid (Synthesis of Diastereomeric Limonene-2-hydroperoxide (trans-6, cis-7) and Limonene-Diepoxide). Additionally, various alcohols (**9**, **12**, **13**, **16**, and **17**) and carvone (**18**) were formed via thermal oxidation of limonene (**1**). Comparing photo- and thermal oxidation, the former mainly yielded hydroperoxides while the latter generated high amounts of epoxides.

Gravimetric Analysis of Oxidized Terpene and Essential Oil Samples. Gravimetric changes occurring upon photo-oxidation of the selected terpenes and essential oils were determined to gain additional information about the degree of oxidation and oxygen turnover. For this purpose, complete terpene monohydroperoxide formation was assumed to calculate factor F (F = molecular weight of terpene−hydroperoxide (M_{R-OOH})/molecular weight of terpene ($M_{terpene}$)) that describes the molecular weight gain due to monohydroperoxide formation. Based on the initial weight of the sample (W_{t_0}), the theoretical weight as terpene−hydroperoxide ($W_{t_{R-OOH}}$) was calculated ($W_{t_{R-OOH}} = F \times W_{t_0}$). The oxidation factor δ, calculated by the measured weight (W_m) divided by $W_{t_{R-OOH}}$, was introduced to get a quick statement about the degree of oxidation (Table 3). An oxidation factor of 1.0 is obtained with complete oxidation to hydroperoxides, while correspondingly lower values go along with incomplete conversion. Herein, the formation of secondary oxidation products was neglected. The fast formation of hydroperoxides in terpenes when using rose bengal is widely described in the literature and can therefore be used as a rapid oxidation test.[16,20] As shown in Table 3, the oxidation factor δ for linalool, linalyl acetate, and β-citronellol is about 1. This is in agreement with the complete degradation of the starting material as described before (Analysis of Oxidized Terpenes). In addition, rose essential oil containing high amounts of β-citronellol showed the same behavior.[37] The remarkably high δ value of citral (δ = 1.15) indicated the formation of multiple oxidized reaction products. In contrast, the high oxidation stability of carvone (**18**) and valencene determined by spectrophotometry was confirmed by lower δ values of 0.84 and 0.91, respectively. Surprisingly, limonene (**1**, δ value 0.93) showed only a slight weight gain although a marked hydroperoxide formation was observed (Analysis of Oxidized Terpenes). Lemon (δ value 0.81) and caraway (δ value 0.83)

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essential oil correspondingly showed low δ values presumably because of high contents of **1** and **18**, respectively. In addition, a lower δ value can also be seen as an indication that other oxidation products (epoxides, alcohols, ketones) have been formed since the degradation of the primary oxidation products has already taken place. From the data obtained, it may also be assumed that single compounds are more susceptible to oxidation (higher δ value) than whole essential oils, which represent multicomponent mixtures (lower δ value) and possibly stabilize themselves by compound interaction. Physically dissolved oxygen can be neglected in the present study because the samples were under vacuum when the solvent was removed.

In conclusion, the presence of hydroperoxides in terpenes and essential oils could be verified with high analytical sensitivity by means of the proposed spectrophotometric method applying Wurster's red. Fresh samples could be easily distinguished from those stored under oxidative conditions. Semiquantitative determination of the peroxide content was achieved using a hydroperoxide standard. The spectrophotometric method presented here was applied and validated for terpenes and essential oils for the first time. The low detection limit, the small sample amount required, and the fast and straightforward application predestine this method for routine analysis. Low-quality oils can be rapidly identified. However, the applicability of the method to additional essential oil matrixes with different terpene compositions should be further investigated. The range of different oxidative follow-up products was demonstrated by comparison of photo-oxidation and thermal oxidation of limonene. Hydroperoxides were predominantly formed upon photo-oxidation, whereas thermal oxidation mostly yielded epoxides. The gravimetric analysis of the oxidation experiments confirmed oxygen uptake and formation of hydroperoxides as detected by means of spectrophotometry and GC/MS.

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Notes

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4.2 Paper 2

Storage-related changes of terpene constituents in caraway (*Carum carvi* L.) under real-time storage conditions

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Storage-related changes of terpene constituents in caraway (*Carum carvi* L.) under real-time storage conditions

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ABSTRACT

Caraway is a widespread agricultural crop that has been utilized by humans since ancient times. It is known as a kitchen spice, medicinal plant in phytotherapy and, due to its high essential oil content, as a fragrant plant and raw material for terpene production. Limonene and carvone, which in turn are used as precursors for the synthesis of further flavorings and fine chemicals, account for more than 90 % of the essential oil. However, the quality of essential oils can quickly deteriorate due to inadequate storage and by external impacts such as heat, oxygen, and light. In the present study, caraway seeds, caraway essential oil as well as pure standards of limonene and carvone were stored in parallel under real-time conditions at 25 °C and 40 °C to assess the effect of the plant matrix and the natural blend of compounds on essential oil stability. GC and HPLC analyses were used to show that the volatile compounds were well protected in the caraway seeds as no significant changes were determined. In comparison, a remarkable loss of limonene (22.4 %) due to oxidation-induced alterations was observed for the pure substance, while carvone contents remained almost constant over the entire storage period. The decline of limonene (9.2 %) as part of the essential oil was much lower while carvone again remained unchanged. The formation of novel minor compounds such as limonene-1,2-epoxide, carveol, and limonene-1,2-diol was mainly due to limonene downstream oxidation and increased with elevated storage temperatures. Volatile compounds were best protected in the intact plant seeds, and storage of the entire essential oil is preferable compared with isolated terpenes due to higher stability as representatively demonstrated for the major compound limonene.

1. Introduction

Caraway (*Carum carvi* L.) is probably one of the oldest spice and medicinal plant utilized by humans. The plant belongs to the *Apiaceae* family and originates from temperate zones in Western Asia and is now mainly cultivated in Poland, Hungary, and Morocco (Teuscher et al., 2006). Commonly, biennial caraway varieties are grown because of higher essential oil contents (3–7 %), but annual varieties also exist (Nemeth, 1999; Solberg et al., 2016). The use of dried caraway seeds (*Fructus carvi*) has a long tradition in folk medicine and lasts until this day. It is a well-known remedy for digestive and gastrointestinal disorders like stomach aches, constipation, flatulence, and nausea (Agrahari and Singh, 2014; Nemeth, 1999). For this purpose, caraway is widely given to newborn children and infants (Nemeth, 1999). Furthermore, caraway seeds are applied to increase maternal milk secretion of breastfeeding women (Agrahari and Singh, 2014). In addition, dried seeds are utilized in the food and beverage industry to aromatize bread, cheese, and alcoholic drinks and is further applied as a seasoning for meat and vegetable meals (Seidler-Lozykowska et al., 2010).

Apart from its use as whole fruit (seeds), caraway essential oil (*Aetheroleum carvi*), produced by hydro-distillation, finds numerous other applications. The annual production volume of caraway essential oil exceeds 30 tons worldwide (Seidler-Lozykowska et al., 2010). The essential oil is mainly composed of *R*-(+)-limonene (35–45 %) and *S*-(+)-carvone (50–60 %), which represent more than 90 % of the entire essential oil. *Cis-/trans*-carveol, dihydrocarvone, dihydrocarveol, myrcene and *α-/β*-pinene are further terpenes found in caraway essential oil (Nemeth, 1999). This oil is used in cosmetics and care products such as toothpaste, mouth wash, soaps, and lotions (Fatemi et al., 2011; Seidler-Lozykowska et al., 2010). Recent studies have shown that caraway

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essential oil exhibits significant antibacterial, antifungal and antioxidant effects (Agrahari and Singh, 2014; Iacobellis et al., 2005). Among its constituents, carvone is of particular importance because of its allelopathic side effects. As such, it is effective as a repellent against insects and is frequently used as sprout inhibitor in postharvest long-term storage of potatoes, replacing harmful chemicals (de Carvalho and da Fonseca, 2006; Nemeth, 1999; Solberg et al., 2016). In addition, *S*-(+)-carvone in caraway seeds is found with an enantiomeric excess of ≥ 98 % (Bouwmeester et al., 1995). And thus, it represents an attractive starting material for the synthesis of new flavor compounds, fine chemicals, and substances with medical relevance (de Carvalho and da Fonseca, 2006). The ubiquitous precursor in limonene biosynthesis is geranyl diphosphate that is cyclized by a monoterpene synthase. Limonene is partly stored in the oil ducts or further oxidized to carvone *via* carveol as an intermediate including the action of both a limonene hydroxylase and a carveol dehydrogenase (Fig. 1) (Bouwmeester et al., 1998). Fatty acids and various lipids, nitrogen containing compounds and fibers are further constituents of caraway seeds (Benkaci-Ali et al., 2014).

Caraway seeds are often transported and stored in jute bags of approximately 50 kg. The recommended transport temperature range of 5 –25 °C should not be exceeded, as this may result in a significant decline of the essential oil content (Gesamtverband der Deutschen Versicherungswirtschaft e.V., 1998). Fehr and co-workers described a storage-related loss of 2.8 % in caraway essential oil within one year (Fehr, 1980). In 1986, El-Wakeil et al. investigated the impact of storage time at 5 °C and 25 °C on the essential oil content of caraway. Oil quality deterioration was mainly caused by the degradation of carvone, which was enhanced at 25 °C compared with 5 °C (El-Wakeil et al., 1986).

The main objective of the present investigation was to compare storage-related changes in caraway seeds, essential oil, and pure terpenes under real-time conditions. Caraway is a suitable essential oil-bearing plant for storage studies, because the size of the seeds allows a homogeneous distribution and aliquots thereof a representative number of individual seeds. For this purpose, the storage study included freshly harvested caraway seeds, essential oil distilled thereof and its main components in isolated form, namely *R*-(+)-limonene and *S*-(+)-carvone. The samples were stored at 25 °C and 40 °C, in order to simulate real conditions during summer months also in hot climate zones. Compound degradation was monitored by gas chromatography coupled to mass spectrometry (GC/MS) and quantitative GC with flame ionization detection (FID). In order to obtain a complete picture of storage-related changes, additional investigations were performed by high performance liquid chromatography coupled to a diode array and a mass selective detector (HPLC-DAD-MS/MS). In addition, characteristic oxidation products were synthesized as reference material for comparison and identified by ^{1}H and ^{13}C NMR analysis.

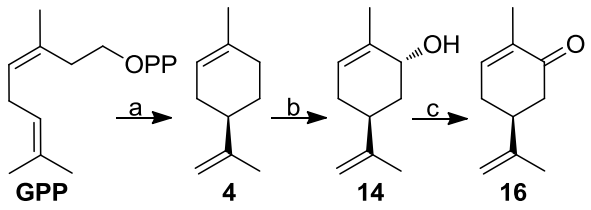

Fig. 1. Biosynthetic pathway of terpene formation in caraway seeds. *R*-(+)-limonene (**4**) is formed from the ubiquitous precursor geranyl diphosphate (**GPP**) and further oxidized to (+)-*trans*-carveol (**14**) which in turn is the reactant for *S*-(+)-carvone (**16**) formation. The enzymes involved in this process are (+)-limonene synthase (*a*), (+)-limonene-6-hydroxylase (*b*) and (+)-*trans*-carveol dehydrogenase (*c*). Figure modified according to Bouwmeester et al. (1998).

2. Materials and methods

2.1. Chemicals

The following chemicals and reference compounds were used: *R*-(+)-limonene (limonene), *S*-(+)-carvone (carvone) (Merck, Darmstadt, Germany); *β*-linalool, linalyl acetate, *β*-citronellol, *β*-myrcene, (*Z*/*E*)-dihydrocarvone, dihydrocarveol, *β*-caryophyllene oxide, *meta*-chloroperoxybenzoic acid, H_2O_2 (30 %) (Sigma Aldrich, Steinheim, Germany); (*Z*/*E*)-carveol (Alfa Aesar-Thermo Fisher, Kandel, Germany); (*Z*/*E*)-limonene-1,2-epoxide, *α*-pinene, *β*-pinene, *γ*-terpinene, *α*-terpineol, *β*-caryophyllene (Carl Roth, Karlsruhe, Germany). Na_2SO_4, NaCl, K_2CO_3, HCl (37 %), silica gel 60 (0.063–0.200 mm), and all solvents used were either of analytical or HPLC grade and purchased from Sigma Aldrich, Merck, and Th. Geyer (Renningen, Germany). Diethyl ether (Et_2O) was distilled in a vacuum rotary evaporator (600 mbar, 38 °C) prior to use to remove the stabilization agent *tert*-butylated hydroxytoluene (BHT). Deionized water applied for aqueous solutions and hydro-distillation was prepared with an Elga Purelab Classic Ultrapure Water System (Elga Labwater, Celle, Germany).

2.2. Storage study

A storage study over a time period of 18 months was performed to determine the quality changes and chemical transformations of caraway (*Carum carvi* L.) seeds, essential oil obtained therefrom and the main terpenes *R*-(+)-limonene and *S*-(+)-carvone, respectively. For this purpose, small bags of untreated cotton gauze (Hans Natur, Süderbrarup, Germany) were sewed with a size of 14 × 8 cm imitating commercial storage of caraway seeds in jute bags (Fig. 2A). Each bag was filled with 80 g air-dried caraway seeds, obtained from the "Sonnenhof" farm (Bad Boll, Germany), grown in Bad Boll in Summer 2018. In addition, aliquots of 1 mL freshly distilled caraway essential oil, pure limonene and carvone were filled into 2 mL clear GC glass vials sealed with a Teflon-coated screw cap. For storage studies, both sample types, *i.e.* bags and vials, were put into two identical desiccators to place them into a 25 °C and 40 °C climate chamber in the dark, respectively. In addition, a 200 mL Erlenmeyer flask filled with a supersaturated potassium carbonate (K_2CO_3) solution was placed in the desiccator to maintain a constant and low humidity to avoid microbial and especially mold growth (Fig. 2A). At intervals of one month, aliquots were taken from both storage temperature regimes. Subsequently, the caraway seeds were distilled as described in Section 2.3, and the obtained essential oil samples were stored after flushing with nitrogen gas at −85 °C for further analysis.

2.3. Preparation of caraway essential oil and evaluation of the oil quantity

Caraway seeds were milled with a Baratza Virtuoso coffee grinder (Baratza, Bellevue, WA, USA) under addition of small portions of dry ice (solid CO_2) to reduce heat development and to displace oxygen thus avoiding premature oxidation. Afterwards, the milled and still frozen seeds were sieved to a particle size of ≤ 1000 µm. Two hundred grams thereof was immediately subjected to hydro-distillation using a 2 L three neck round bottom flask filled with 1100 mL of ultrapure water and equipped with a 30 cm distillation bridge. The system was heated with an IKA RCT Standard heating plate (IKA, Staufen, Germany) at 180 °C and stirred at 260 rpm for 240 min using a KPG stirrer. The distilled caraway essential oil (***D***) was separated from the aqueous phase using a separating funnel, and aliquots of 1 mL were placed in 2 mL clear glass vials for storage studies.

Monthly samples of the caraway seeds, stored in cotton bags as described before, were distilled into fresh essential oil (***M***). Herein, milled seeds (sieved to ≤ 1000 µm as specified above) were distilled by means of a Clevenger apparatus according to the European Pharmacopoeia but without xylene addition (European Pharmacopoeia, 2017a).

Fig. 2. Caraway seeds in sewed linen bags (14 × 8 cm) stored in a desiccator (**A**). Carvone stored for 0, 3, 6 and 12 months at 40 °C in the dark (**B**). Limonene stored for 0, 3, 6 and 12 months at 40 °C in the dark (**C**).

An aliquot (10.0 ± 0.1 g) was weighed into a 500 mL round bottom flask, 200 mL deionized water and glass boiling beads were finally added to avoid boiling delays. The system was then heated to 170 °C for 90 min. The essential oil yield was determined volumetrically by the unit attached to the apparatus. Thereafter, the oil samples were flushed with nitrogen and stored at -85 °C prior to GC and HPLC analyses.

In addition, a commercially available caraway essential oil (***C***) with a well-defined history of twelve months since distillation was obtained from Naturamus (Aichelberg, Germany) and also placed in half-filled vials into the desiccators.

2.4. Synthesis of carvone-1,2-epoxide, limonene-1,2-epoxide, and limonene-1,2-diol

Carvone-1,2-epoxide (**18**) was synthesized as a diastereomeric mixture according to Nagasawa et al. (2019). In brief: A solution of 5.0 g (33.3 mmol) *S*-(+)-carvone in 83 mL of methanol was cooled to −15 °C and 2.5 mL of an aqueous 4 M NaOH solution was added. Subsequently, 5.0 mL (43 mmol) H_2O_2 (30 % in H_2O, w/w) was added dropwise to the reaction mixture. Thereafter, the solution was stirred at 800 rpm and allowed to warm up to −5 °C within 2 h. Additional 2.5 mL (22 mmol) of H_2O_2 were added and warmed up to 0 °C. Afterwards, 8 mL hydrochloric acid (1 N, 8 mmol) and 5.0 g $Na_2S_2O_3 \times 5\ H_2O$ (20 mmol) was added and stirred for 1 h. Subsequently, the reaction was quenched by adding 100 mL deionized H_2O and extracted with 3 × 80 mL Et_2O. After drying over Na_2SO_4 the Et_2O extracts were evaporated *in vacuo* to approximately 20 mL, washed again with 150 mL H_2O and extracted with *n*-pentane : Et_2O (1 : 1; 3 × 80 mL). The combined organic phases were dried (Na_2SO_4) and evaporated *in vacuo*. The crude product (4.5 g, 27 mmol, 81 % yield) was obtained with a GC purity of 98 % and was used for analysis without further purification. The ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) spectra were in accordance with literature data (Feng et al., 2008; Nagasawa et al., 2019). MS (EI) *m/z* (%): 166 (M^+, 1), 123 (27), 109 (13), 85 (26), 67 (50), 43 (100).

Limonene-1,2-epoxide (**9**, **10**) was synthesized *via* Prileschajew reaction from limonene and subsequent alkaline hydrolysis to the corresponding diol (limonene-1,2-diol, **20**). In brief: 4.04 g *R*-(+)-limonene (29.6 mmol) was dissolved in 100 mL dichloromethane (DCM) and cooled to −15 °C. Afterwards, a solution of 6.04 g (35 mmol) *m*-chloroperoxybenzoic acid (*m*-CPBA) in 50 mL DCM was added. After 1 h the reaction mixture was warmed to 20 °C and stirred for another 60 min at 800 rpm. The reaction was stopped by adding 100 mL H_2O, and extraction with DCM (2 × 100 mL) was performed. The combined organic layers were washed with 150 mL H_2O, dried over Na_2SO_4 and evaporated to dryness (Gattermann, 1983). A white solid was obtained, washed with *n*-pentane (2 × 50 mL) and the solvent again evaporated *in vacuo*. Subsequently, the crude product was purified by column chromatography on silica gel (30 g). Gradient elution was started with 100 % *n*-hexane, followed by stepwise addition of Et_2O to a final concentration of 80 % (v/v) Et_2O in *n*-hexane. Individual fractions were analyzed by GC, and relevant fractions containing *cis-/trans*-limonene-1,2-epoxide were combined and stored at −30 °C after evaporation *in vacuo* (2.83 g yield, 63 % of the theory). GC/MS spectra were compared with an authentic limonene-1,2-epoxide reference standard. *cis*-limonene-1,2-epoxide (43 %; **9**) MS (EI) *m/z* (%): 152 (M^+, 5), 137 (33), 109 (37), 93 (45), 67 (99), 43 (100). *trans*-limonene-1,2-epoxide (57 %; **10**) MS (EI) *m/z* (%): 152 (M^+, 3), 108 (75), 94 (100), 79 (67), 67 (71), 43 (78) (Blair et al., 2007).

A portion of 2.00 g (13.1 mmol) of the synthesized limonene-1,2-epoxide was dissolved in a H_2O : ethanol mixture (100 mL, 70 : 30, v/v) and the pH value was adjusted to 10 by addition of NaOH solution (4 N). Then, the reaction mixture was stirred at 800 rpm for 20 h and subsequently acidified to pH 6 by adding HCl (1 N). Afterwards, the solution was extracted with ethyl acetate (EtOAc, 3 × 70 mL), the organic solvent dried over Na_2SO_4 and evaporated *in vacuo*. Afterwards, the crude product was purified by column chromatography on silica gel (30 g) applying an EtOAc/*n*-hexane gradient from 1:99 to 100:0 (*v/v*). Individual fractions were analyzed by GC, and relevant fractions containing (1*S*,2*S*,4*R*)-limonene-1,2-diol (main isomer ≥ 98 %; **20**) were combined and stored at −30 °C after evaporation *in vacuo* (0.99 g, 44.3 % of the theory). The ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data were in good accordance with the literature (Blair et al., 2007). MS (EI) *m/z* (%): 170 (M^+, 1), 152 (30), 108 (36), 82 (29), 71 (100), 43 (74).

2.5. Determination of the water content of caraway seeds

The water content of dried caraway seeds was determined according to the European Pharmacopoeia method (European Pharmacopoeia, 2017b). In brief: 10.0 g of milled (≤ 710 µm) caraway seeds were distilled with toluene at 165 °C for 2 h using a distillation apparatus as described in the European Pharmacopoeia. After complete distillation and separation of the aqueous phase from toluene, the water content was determined volumetrically. The analysis was carried out in duplicate, and the water content was calculated based on a seed weight of 1000 g. Herein, an average water content of 82.1 mL/kg was determined for the caraway seeds used in the current study.

2.6. Analytical characterization

To get a comprehensive picture of aging phenomena and the formation of follow-up products, the essential oils and terpenes were successively analyzed by GC and HPLC. Quantitation of the components was carried out using a FID coupled to a gas chromatograph. Sensory evaluation was also performed, as smell is a highly important quality criterion in the field of essential oils.

2.6.1. Gas chromatography (GC/MS and GC/FID)

Gas chromatographic experiments were performed with a Perkin Elmer Clarus 500 gas chromatograph equipped with two split/splitless injectors coupled to a Perkin Elmer Clarus 500 Mass Selective Detector with a quadrupole mass filter and a flame ionization detector (FID), respectively. Data acquisition and control of the system was achieved with TurboMass software, version 6.1.2 (Perkin Elmer), and TotalChrom, version 6.3.2 for MS and FID analysis, respectively. Injection volume was 1.0 µL with a split ratio of 1:20 for samples in methyl *tert*-butyl ether (MTBE). Helium was used as a carrier gas at a constant flow of 1.0 mL/min. The analytical column used for MS and FID analyses was coated with 5 % phenyl 95 % methyl polysiloxane (60 m × 0.25 mm i.d., 0.25 µm d_f, ZB-5 ms, Phenomenex, Torrance, CA USA).

The following temperature program was used for both MS and FID analyses: initial temperature 60 °C, isothermal hold for 4 min at 60 °C, raise to 240 °C (6 °C/min) followed by a ramp to 280 °C (8 °C/min) and 8 min isothermal hold. The total run time was 47 min. Source and transfer line temperatures were set to 180 and 220 °C, respectively. Mass spectra were recorded in the electron ionisation (EI) mode over a scan range of *m/z* 30−300. Analysis was performed in triplicate and data evaluation of the chromatograms was done with TurboMass Data Analysis Application and TotalChrom workstation (Perkin Elmer). Compound assignment was based on mass spectra and linear retention indices (LRIs) according to van den Dool and Kratz (1963) as found in the NIST database as well as by using authentic reference standards if available.

2.6.2. High performance liquid chromatography (HPLC-DAD-APCI-MS/MS)

The following method was modified according to Turek and Stintzing (2011). The essential oil and terpene samples were dissolved in appropriate concentrations in ethanol prior to HPLC analysis. An Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) was used, equipped with a binary pump, a micro-vacuum degasser, an auto sampler, a thermostatic column compartment, and a UV/Vis diode array detector. For chromatographic separation a Modulo-Cart MS Uptisphere ODB 3UM reversed phase (RP) column (150 mm × 2.1 mm i.d.) with a particle size of 3.0 µm from Interchim (Montluçon, France) combined with a C_{18} security guard column (4 mm × 2.0 mm i.d.) was chosen. Separation was performed at 20 °C with a flow rate of 0.210 mL/min using purified water (mobile phase A) and acetonitrile/formic acid 99.9:0.1 (mobile phase B) as eluent. Starting with 30 % B for 2 min, a linear gradient was followed to 45 % B at 10 min, then increasing to 60 % B at 25 min, and finally to 100 % B at 50 min, continuing for 10 min, before re-equilibration to starting conditions within 10 min. The injection volume of each sample was 10 µL. Simultaneous monitoring was performed at 200, 205, 210, 220 and, 310 nm (band width and slit 4 nm), respectively. Additionally, UV–vis spectra were recorded from 190 to 720 nm. The LC system was connected in series to an HCT Ultra ion trap mass selective detector (Bruker Daltonik, Bremen, Germany) with an atmospheric pressure chemical ionization (APCI) source operating in the positive ionization mode by applying the following parameters: capillary voltage, −3000 V; corona current, 4000 nA; skimmer voltage, 30 V; capillary exit voltage, 116.7 V; and trap drive, 22.4. Nitrogen was used as drying gas with a flow rate of 5.0 L/min at 200 °C. The vaporizer temperature was 250 °C and the nebulizer pressure was set at 40.0 psi. Full-scan mass spectra of the eluates were recorded from *m/z* 15 to 300. To obtain further structural information, collision-induced dissociation (CID) experiments were performed in the auto MS/MS mode. Data acquisition and control of the system was achieved with Agilent ChemStation and Esquire Control Software version 6.1.

2.6.3. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded in $CDCl_3$ at 600 (^{1}H) and 150 MHz (^{13}C) using a 600 MHz Bruker Avance III HD NMR spectrometer. Chemical shifts are reported in δ [ppm] and refer to residual solvent signals of $CDCl_3$ (^{1}H: 7.27 ppm; ^{13}C: 77.00 ppm). NMR spectra were evaluated using SpinWorks 3.1.7 software (Copyright 2010, K. Marat, University of Manitoba, Winnipeg, MB, Canada).

3. Results and discussion

3.1. Essential oil content of caraway seeds

The overall amount of essential oil obtained from the seeds by a Clevenger apparatus (Section 2.3) remained virtually unchanged over the entire observation period. The average yield of 91 mL essential oil per kg seeds at the beginning of storage was very high which is assumed to be a consequence of the water deficit in the fields during hot summer months in 2018. As described by Laribi et al., moderate water deficits may increase essential oil yield in *Carum carvi* seeds (Laribi et al., 2009). Storage of the seeds at 25 °C and 40 °C resulted in very similar essential oil yields. Over the time range of 15 months slight losses of approximately 10 mL oil/kg seeds per year was observed which is modest regarding the volatility of the essential oil components. This indicates that the essential oil was very well kept in the seeds. The oil ducts of caraway seeds are well covered by a protecting periderm (Nemeth, 1999). Stabilization might also be achieved by glycosylation of terpenes containing a hydroxyl group as described by Yazaki and co-workers (Morcol et al., 2020; Yazaki et al., 2017). For limonene and carvone the precursors geranyl diphosphate and carveol may undergo glycosylation here, respectively (Bouwmeester et al., 1998). Slight fluctuations upon storage may be explained by small sample amounts (10.0 g) used for distillation (data not shown) as described in the European Pharmacopoeia or inhomogeneous particle size of the milled seeds (European Pharmacopoeia, 2017a, 2017b).

3.2. Olfactory and visual assessment of samples upon storage

Being important quality criteria, the stored caraway essential oil samples were assessed upon storage in terms of their visual and olfactory traits prior to instrumental analysis. As illustrated in Fig. 2B, a remarkable change in the color of pure carvone from clear transparent to light brown could be observed during storage. Also, stored caraway essential oil developed a yellow-brownish color that could be detected after four months of storage at 40 °C and after seven months at 25 °C. Since limonene samples did not show visible color evolution upon storage (Fig. 2C), the color change of caraway essential oil seemed to be only due to carvone aging. However, this could not be further verified in the present study. In contrast, limonene revealed a pronounced deterioration in odor due to the loss of its fresh, fruity, and citric smell (Schieberle and Grosch, 1989). Already after three months a pungent and artificial note was perceived. Also, the freshly distilled caraway essential oil showed a musty sulfurous odor in the beginning which, however, was lost after a short storage time (3 months) and then corresponded to the well-known caraway smell. Potent odorous sulfur containing terpenes have been described for various essential oils, possibly originating from the thermal breakdown of certain S-containing amino acids (*e.g.*, cysteine and methionine) initiated during the distillation process (Berger, 2007).

3.3. Quantitative analysis of carvone and limonene in caraway essential oil by GC/FID

The freshly distilled caraway essential oil (***D***) had an average content of 47.4 % limonene and 50.5 % carvone, thus representing 98 % of the entire oil at the beginning of storage (Fig. 3A and Table 1). Similarly, both compounds contributed ~96 % to the commercial oil (***C***, Fig. 3B). These values were within the range of previous reports, although both origin and genotype generally have a strong impact on the composition of essential oils (Nemeth, 1999). For instance, limonene (carvone) ranged from 16.2 % (14.0 %) to 70.7 (77.4 %) in different caraway essential oil samples (Laribi et al., 2013; Solberg et al., 2016). In accordance with these findings, the limonene range of the commercial caraway essential oil (***C***, Fig. 3B) was below that of the freshly distilled caraway essential oil (***D***, Fig. 3A).

In the latter (***D***), slight fluctuations of limonene and carvone were detected in the first four months of storage, which were even more pronounced at 25 °C compared with 40 °C (Fig. 3A). As this was also observed in the commercial caraway essential oil sample (***C***) from 2017, the constantly increased temperature by starting the storage also seems to be of relevance. It is assumed, that in the beginning carvone was replicated from limonene by thermal degradation. This pathway *via* an enzymatic reaction has previously been described in the literature (Fig. 1) (Bouwmeester et al., 1998). After that period (month 1–4), limonene contents constantly decreased at both temperature regimes (*i.e.* 0.5 %/month on average in case of 40 °C, Fig. 3A). At the end of the storage experiment, the limonene content at the lower temperature (25 °C) was about 4 % higher compared with 40 °C (Fig. 3A). Apparently, storage at lower temperatures slowed down the degradation of substances prone to oxidation such as limonene in the present work (Turek and Stintzing, 2013) such as limonene in the present work . The overall loss of limonene amounted to 9.2 %

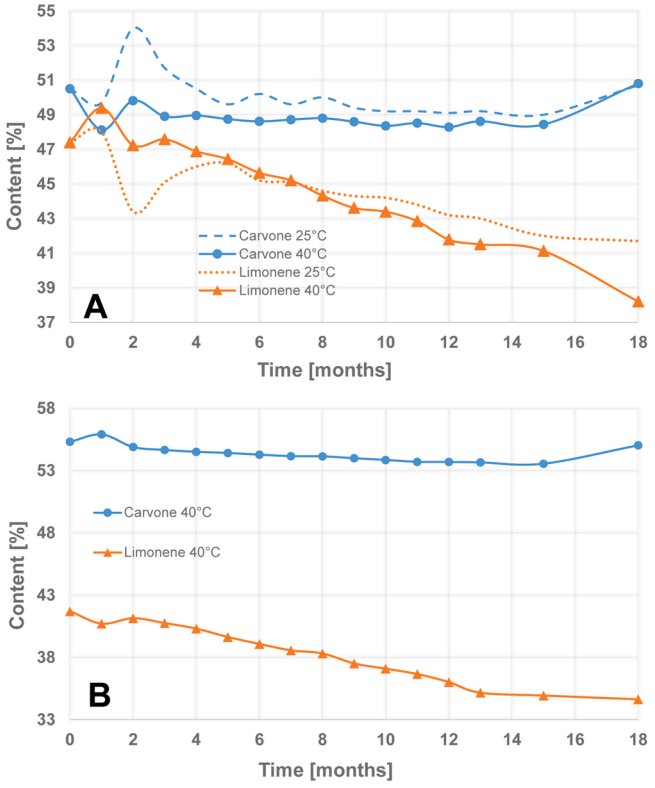

Fig. 3. Relative content (% of total GC/FID peak area, $n = 3$) of limonene and carvone in caraway essential oil ***D*** stored at 25 °C and 40 °C (**A**). Relative content (% of total GC/FID peak area, $n = 3$) of limonene and carvone in commercial caraway essential oil ***C*** from 2017 stored at 40 °C (**B**). Both samples stored in the dark.

upon storage at 40 °C, which markedly changed the profile of the caraway essential oil. However, the characteristic odor remained unaffected because it was still dominated by carvone. Carvone showed a noticeably greater stability at both storage temperatures. Its overall content remained virtually unchanged over the whole time, which represents an important finding, since carvone is a quality marker of caraway essential oils used for medicinal and industrial purposes (de Carvalho and da Fonseca, 2006). In contrast to our investigations, El-Wakeil et al. (1986) described carvone losses over a period of twelve months considering different packaging materials and storage at both 5 °C and 25 °C. Interestingly, the initial proportion of carvone in the essential oil used in that study amounted to > 80 % (El-Wakeil et al., 1986). However, the present study even revealed a constant carvone content after a storage period of 15 months (Fig. 3A). Hence, slight carvone losses, as indicated by the color change during storage of the neat compound (Fig. 1B), were compensated by its formation upon the degradation of minor components derived from limonene, especially limonene-epoxides and carveol. This is in line with earlier reports indicating carvone to be formed from these compounds as a result of continued oxidation processes (Casuscelli et al., 2004; de Carvalho and da Fonseca, 2006). Remarkably, the proportion of the two major compounds of the commercial essential oil (***C***) resembled those of the freshly distilled essential oil after 18 months of storage (Fig. 3A, B). Also, storage of the commercial essential oil (***C***) started with a decrease in limonene (41.4 %) and an increase in carvone (55.3 %) (Fig. 3B), followed by a drop in limonene (0.4 % per month on average) and only slight variations in the carvone content (Fig. 3B). Similar changes (0.5 % and 0.4 % per month in samples (***D***) and (***C***), respectively) indicated that the decline in limonene contents will continue on a similar level with time, irrespective of the sample. This is in agreement with a high susceptibility of limonene to oxidation with a trend to degrade almost completely over time as reported in other studies (Bitterling et al., 2020; Schieberle et al., 1987). Similarly, also carvone proportions remained almost stable in both essential oil samples ***D*** and ***C***. A slight carvone loss was due to the formation of carvone-1,2-epoxides as detected by GC/MS measurements reaching a maximum of 0.5 % after 15 months. *Vice versa*, an increase of carvone was also associated with (oxidative) formation of limonene transformation products (*e.g.* carveol). However, by quantitation *via* GC area normalization the formation and degradation of compounds is related to each other. In contrast to samples ***C*** and ***D*** which were distilled from freshly harvested plant material and stored over 18 months, a further set of samples was obtained by distilling essential oil from stored samples (sample ***M***, Section 2.3). Interestingly, changes in the composition of the latter from the start until month 18 were negligible. In particular, fluctuations of carvone and limonene proportions were marginal and amounted to only 4 % without any identifiable trend (data not shown). Apparently, these variations are mainly due to minute fluctuations in the distillation process. Hence, the terpenes were well protected in intact caraway seeds even when stored at 40 °C.

3.4. Quantitative analysis of pure carvone and limonene by GC/FID

In comparison to the multicomponent blend of an essential oil, the neat terpenes limonene and carvone were subjected to the same storage conditions to gain further knowledge on alteration processes of mono substances under mild conditions (Fig. 4). Storage of pure limonene at 25 °C under the aforementioned conditions (half-filled clear glass) resulted in a decrease from 96.6 % at the beginning to 79.1 % after 18 months, corresponding to a total loss of 17.5 percentage points. This overall decrease of compounds was more pronounced at 40 °C (22.4 percentage points). Interestingly, the decline of the terpene contents was found to follow a very linear way as illustrated in Fig. 4. An r^2 of 0.992 and of 0.994 was calculated for limonene degradation at 25 °C and 40 °C, respectively.

In comparison, the loss of pure carvone within one year amounted to only 2.3 % (25 °C) and 4 % (40 °C) (Fig. 4). Similar to pure limonene, the degradation of pure carvone was linear at both temperatures ($r^2 = 0.936$ and $r^2 = 0.996$ for 25 °C and 40 °C, respectively, Fig. 4). It was assumed,

Table 1
Compounds and composition (% of total peak area GC/FID, $n = 3$) of caraway essential oil after distillation of fresh seeds (t0) and storage of the same at 40 °C for 18 months (t18) in the dark.

No.	Compound	Composition [%]		RI_a	RI_b	M_c	Identification	Reference
		t0	t18					
1	*α*-pinene	0.04 ± 0.00	0.04 ± 0.00	933	934		RI, GC/MS[a]	Laribi et al., 2013
2	*β*-pinene	0.08 ± 0.00	0.02 ± 0.00	969	977		RI, GC/MS[a]	Benkaci-Ali et al., 2014
3	*β*-myrcene	0.46 ± 0.00	0.04 ± 0.00	980	991	x	RI, GC/MS[a]	Laribi et al., 2013
4	*R*-(+)-limonene	47.4 ± 0.03	38.18 ± 0.10	1027	1028	x	RI, GC/MS[a]	Benkaci-Ali et al., 2014
5	*β*-ocimene	0.07 ± 0.00	n.d.	1034	1040		RI, GC/MS	Benkaci-Ali et al., 2014
6	*γ*-terpinene	0.02 ± 0.00	n.d.	1049	1054		RI, GC/MS[a]	Benkaci-Ali et al., 2014
7	*β*-linalool	0.05 ± 0.00	0.07 ± 0.00	1085	1100		RI, GC/MS[a]	Laribi et al., 2013
8	*trans-p*-mentha-2,8-diene-1-ol	0.03 ± 0.00	0.49 ± 0.00	1113	1123	x	RI, GC/MS	Simic et al., 2008
9	*cis*-limonene-1,2-epoxide	0.02 ± 0.00	0.46 ± 0.00	1124	1135	x	RI, GC/MS[a]	Iacobellis et al., 2005
10	*trans*-limonene-1,2-epoxide	0.07 ± 0.00	0.86 ± 0.01	1127	1136	x	RI, GC/MS[a]	Laribi et al., 2013
11	*α*-terpineol	0.02 ± 0.00	0.11 ± 0.02	1186	1189		RI, GC/MS[a]	Laribi et al., 2013
12	*cis*-dihydrocarvone	0.13 ± 0.00	0.46 ± 0.04	1189	1193	x	RI, GC/MS[a]	Simic et al., 2008
13	*trans*-dihydrocarvone	0.10 ± 0.00	0.53 ± 0.06	1197	1201	x	RI, GC/MS[a]	Simic et al., 2008
14	*trans*-carveol	0.10 ± 0.01	0.70 ± 0.03	1210	1217	x	RI, GC/MS[a]	Simic et al., 2008
15	*cis*-carveol	0.16 ± 0.00	0.56 ± 0.12	1225	1229	x	RI, GC/MS[a]	Simic et al., 2008
16	*S*-(+)-carvone	50.5 ± 0.03	50.76 ± 0.18	1244	1245		RI, GC/MS[a]	Iacobellis et al., 2005
17	Dihydrocarveol	0.04 ± 0.00	0.08 ± 0.00	1251	1253		RI, GC/MS[a]	Laribi et al., 2013
18	Carvone-1,2-epoxide	n.d.	0.39 ± 0.00	1265	1263	x	RI, GC/MS[a]	Benkaci-Ali et al., 2014
19	Perillaldehyde	0.22 ± 0.00	0.22 ± 0.00	1270	1272		RI, GC/MS	Laribi et al., 2013
20	Limonene-1,2-diol[c]	n.d.	1.28 ± 0.01	1334	1321	x	RI, GC/MS[a]	Hamm et al., 2005
21	n.i.[c]	n.d.	0.16 ± 0.01	1353		x	GC/MS	
22	n.i.[c]	n.d.	0.31 ± 0.00	1364		x	GC/MS	
23	3-Hydroxy-dihydrocarvone[c]	n.d.	0.24 ± 0.01	1400		x	GC/MS[b]	
24	*β*-caryophyllene	0.14 ± 0.00	0.10 ± 0.00	1420	1419		RI, GC/MS[a]	Laribi et al., 2013
25	Germacrene-D	0.02 ± 0.00	0.03 ± 0.01	1481	1480		RI, GC/MS	Laribi et al., 2013
26	*β*-caryophyllene oxide	0.01 ± 0.00	0.11 ± 0.00	1590	1576		RI, GC/MS[a]	Benkaci-Ali et al., 2014
	% of identification	99.7 %	96.2 %					

RI_a, retention indices relative to C_8-C_{20} *n*-alkanes.
RI_b, reference indices.
M_c, marker-compound for alteration process.
n.d., not detected.
n.i., not identified.
±, standard deviation ($n = 3$).
[a] Identified by comparison with reference standard.
[b] Tentatively identified.
[c] Detected for the first time in caraway essential oil.

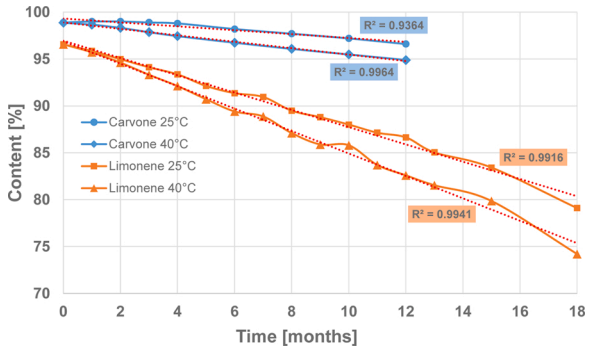

Fig. 4. Quantitative analysis (% of total GC/FID peak area, $n = 3$) of the pure terpenes limonene and carvone stored in the dark over a time range of 12 and 18 months in half-filled clear glass vials at 25 °C and 40 °C.

that lacking interactions and antioxidant effects of further oil components led to an accelerated degradation and increased the overall loss of the pure terpenes. As described by Samojlik et al. (2010), various substances of caraway essential oil, namely carvone and carveol, exhibit a strong antioxidant capacity. Low amounts were sufficient to neutralize H_2O_2 ($IC_{50} \leq 2.5$ µL/mL) or the DPPH (IC_{50} 4.1 µL/mL) radical (Samojlik et al., 2010). For this reason, oxidation processes can therefore successfully be reduced and delayed, possibly explaining the differing degradation rates of carvone and limonene either as pure substance or in the essential oil matrix under identical conditions. According to GC/MS and GC/FID analyses, the main degradation products of pure carvone after twelve months of storage were carvone-1,2-epoxide (**18**, 0.9 %; Table 1) and 3-hydroxy-dihydrocarvone (3-hydroxy-2-methyl-5(prop-1-en-2-yl)cyclohexanone; **23**, 1.5 %). Noteworthy, **23** is described here for the first time in caraway essential oil. This is particularly interesting, because distinct anti-inflammatory and antinociceptive activities of **23** have been observed in rats and mice (de Sousa et al., 2010).

3.5. Quantitative analysis of minor compounds in caraway essential oil and limonene by GC/FID

The content of minor components in freshly distilled caraway essential oil (***D***) strongly depended on the degree of maturation or aging as illustrated in Fig. 5A and Table 1. In many cases, their contents increased upon storage of caraway oil ***D*** (Fig. 5A; Table 1, M_c, marked with an "x"). Virtually the same compounds were also detected in stored pure limonene. In this case, seven of these minor components showed a remarkable increase throughout storage at 40 °C (Fig. 5B).

While *cis*- (**9**) and *trans*-limonene-1,2-epoxide (**10**) (for these and other structures see Fig. 6) were not detectable in the freshly distilled essential oil (***D***), *cis*- (**15**, 0.16 %) and *trans*-carveol (**14**, 0.10 %) were present in relatively high concentrations (Table 1). During the first months of storage, both epoxides (**9**/**10**) rapidly increased whereas *cis*-/*trans*-carveol (**14**/**15**) and *trans-p*-mentha-2,8-dien-1-ol (**8**) showed a very slow rise (Fig. 5A). After nine months, the share of **9** reached a plateau (0.79 %) in ***D*** followed by a drop after twelve months. The *trans*-isomer **10** showed a similar progression but increase continued until the plateau was reached after 15 months at 1.05 %. Arguably, the

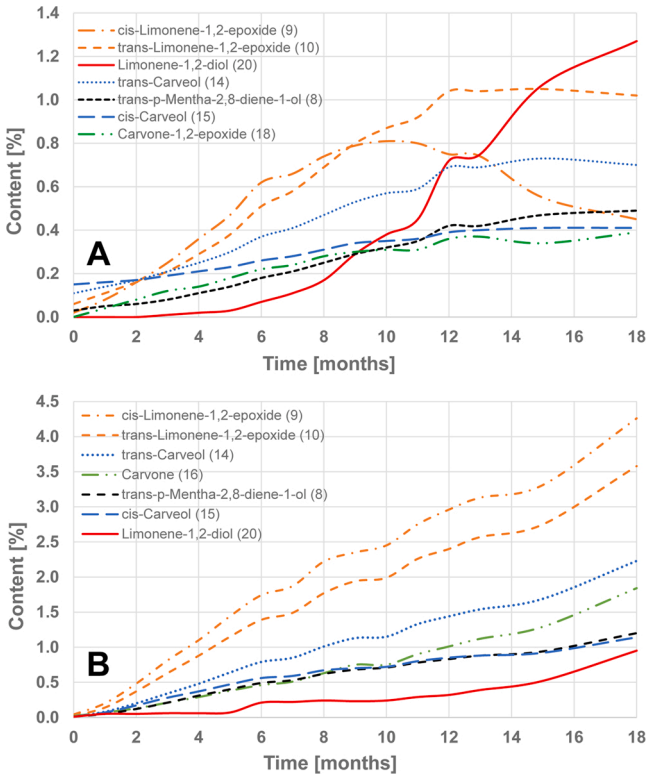

Fig. 5. Increase of minor components upon storage at 40 °C of freshly distilled caraway essential oil (***D***) for 18 months in the dark (% of total GC/FID peak area, $n = 3$) (**A**). Increase of minor components in *R*-(+)-limonene stored at 40 °C for 18 months in the dark (% of total GC/FID peak area, $n = 3$) (**B**).

delayed curve may indicate a depletion of **10** at even longer storage times. After a lag time of ~5 months without detection, the amount of limonene-1,2-diol (**20**) sharply increased in the stored caraway essential oil ***D*** which describes an interesting finding, since other downstream products were formed at a significantly lower rate. The identity of **20** was confirmed by GC/MS data, which were compared with those of an authentic reference standard. Most likely, **20** was formed by degradation of both epoxides (**9**/**10**; Fig. 6), since decomposition of epoxides to diols is a well-known secondary reaction (Casuscelli et al., 2004). Limonene-1,2-diol showed highest concentrations (1.27 %) in the essential oil at the end of storage. Interestingly, not only limonene, but also the minor terpene *β*-myrcene was strongly degraded in ***D*** upon storage (Table 1). Apparently, also *β*-ocimene and *γ*-terpinene were degraded because they were detected at traces at the start but not anymore at the end of the storage study (Table 1). Possible degradation products may be **21** (0 %→0.16 %) and **22** (0 %→0.31 %). However, unambiguous assignment was not possible in this study.

To get a deeper insight into the formation of minor compounds, they were also studied in the storage experiment with pure limonene (Fig. 5B). Detection of the same main degradation products as in caraway essential oil ***D*** confirmed both the instability of limonene and the formation of oxidation products thereof. A permanent increase of seven follow-up products was observed, which was most pronounced for limonene epoxide reaching a maximum after 18 months of 4.26 % for the *cis*-isomer (**9**) and 3.58 % for the *trans*-isomer (**10**). In addition, both carveol isomers (**14**/**15**) as well as *trans-p*-mentha-2,8-dien-1-ol (**8**) and carvone (**16**) reached maximum levels between 1 and 2 %. Interestingly, limonene-1,2-diol (**20**), resulting from the degradation of limonene-1,2-epoxide (**9**/**10**), showed lowest concentrations (0.95 %), which indicates that decomposition of **9** and **10** did not take place yet, which is in agreement with their high amounts (see above). Formation of limonene-1-hydroperoxides and limonene-2-hydroperoxides (Fig. 6A) as primary

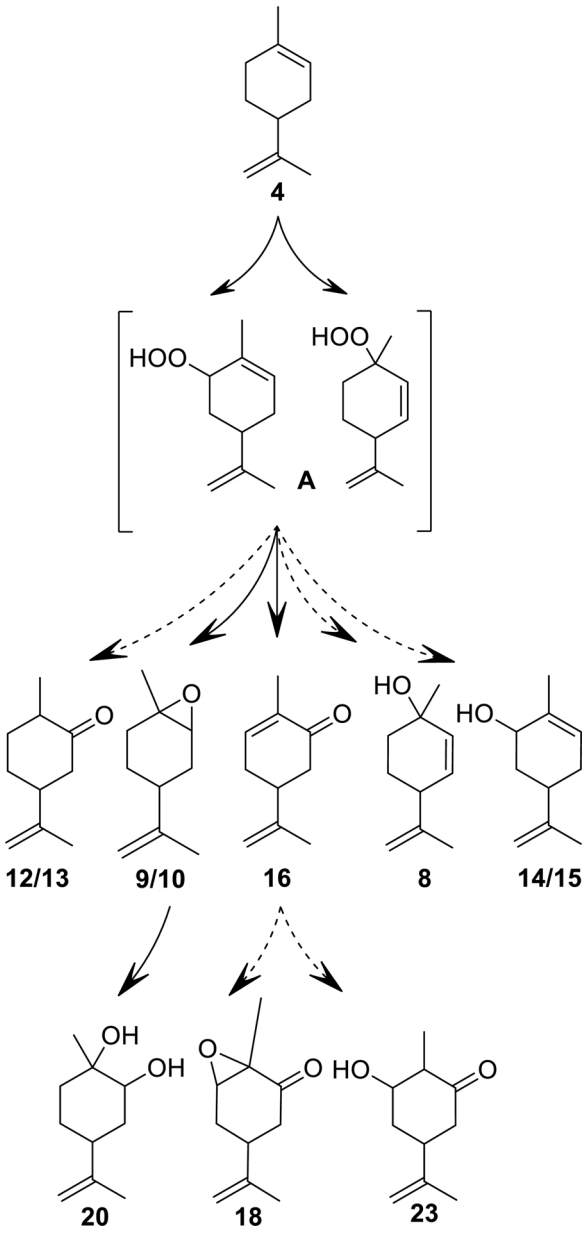

Fig. 6. Structures of main compounds formed during storage of caraway essential oil identified by GC/MS. Plain lines (-) illustrate main reaction pathways. The formation of minor compounds is illustrated by dotted lines (–). The formation of hydroperoxide intermediates (**A**) was not detected here. **4**, *R*-(+)-limonene; **12**/**13**, *cis- trans*-dihydrocarvone; **9**/**10**, *cis- trans*-limonene-1,2-epoxide; **16**, *S*-(+)-carvone; **8**, *trans-p*-mentha-2,8-diene-1-ol; **14**/**15**, *trans- cis*-carveol; **20**, limonene-1,2-diol; **18**, carvone-1,2-epoxide; **23**, 3-hydroxy-dihydrocarvone.

oxidation products as described by Clark et al. could not be confirmed in this study. This may indicate that thermal oxidation played a major role, because epoxides were shown to be rather formed under such conditions, whereas hydroperoxides are mainly generated in the presence of light (Bitterling et al., 2020). Accordingly, ketones (**12**/**13**, **16**), epoxides (**9**/**10**), and alcohols (**8**, **14**/**15**) were the first intermediates present in considerable quantities. Formation of **20** from **9** and **10** could be identified as main reaction pathway, whereas **9** and **10** were directly formed from limonene due to thermal load (Fig. 6). Oxidation products of carvone could also be detected as compounds **18** and **23**. In summary, limonene stored in pure form produced significantly higher contents of oxidation products than the essential oil and the amount of oxygen

absorbed by the terpenes increased over time (Fig. 6).

3.6. HPLC-DAD-APCI-MS/MS analysis of caraway essential oil and limonene

HPLC analysis represents a worthwhile option for complementary essential oil characterization (Turek and Stintzing, 2011). In agreement with that, additional storage-related changes in caraway essential oil composition could be verified by HPLC-DAD (Fig. 7). Especially, the so-called "fingerprint region" that is specific for an essential oil and defined by diverse minor components (Turek and Stintzing, 2011) featured a number of oxidation products (**9**, **10**, **14**, **15**, **18**, **23**) in caraway essential oil ***D*** after 15 months, which were not detected at the beginning of storage (Fig. 7A, B). An overlay of the fingerprint region of stored essential oil ***D*** and pure limonene samples (Fig. 7D) confirmed that the key oxidation products were mainly obtained from degradation of limonene (Fig. 7C). Noteworthy, neither stored samples of essential oils nor pure standards of limonene (**4**) and carvone (**16**) indicated the presence of hydroperoxides *via* HPLC which is well-suited for detection of the latter due to milder temperature conditions (Turek and Stintzing, 2013; Rudbäck et al., 2013). Furthermore, the two main oxidation products of the terpenes, limonene-1,2-epoxide (**9**,**10**) and carvone-1,2-epoxide (**18**), could also be detected by HPLC-APCI-MS/MS (Fig. 7E, F). In addition, relevant changes during the storage of pure carvone could not be detected by HPLC. This also confirmed the high storage stability of this terpene both as pure substance and in the essential oil.

All in all, HPLC measurements confirmed the results obtained by GC analyses and supported the hypothesis of accelerated hydroperoxide formation only under the impact of light (Bitterling et al., 2020).

4. Conclusions

The comparative storage study including caraway seeds, essential oil thereof and the pure reference terpenes limonene and carvone provided valuable insights into terpene oxidation in the plant matrix, multicomponent mixtures, and pure substances under identical storage conditions for the first time. Essential oil components were well protected inside the plant seeds. The overall loss of essential oil was marginal during storage over 18 months and the compound profile remained unchanged. Also, storage of the essential oil at mild temperatures and under exclusion of light was found to be adequate for several months or even up to years. Carvone showed nearly no degradation under the given conditions which is of high relevance since this is the characteristic flavor substance in caraway essential oil used for several industrial purposes. In contrast, pure limonene exhibited strong degradation, and formation of several oxidation products was monitored. Limonene-epoxides may be used as a marker substance for the initial stage of aging, whereas limonene-1,2-diol formation indicates an even longer storage period and temperature load. This was mostly pronounced for storage of the pure terpene. The storage of essential oils is therefore more favorable than that of pure substances, as mutual stabilization and antioxidant effects of certain components can effectively slow down oxidation processes. The yellow-brownish color of caraway essential oil is mainly due to negligible carvone polymerization and is compensated by its formation from limonene. Color formation and carvone loss can be delayed significantly by storage at lower temperatures. To achieve highest oil qualities, it is advisable to store the entire seeds until further processing. If this is not possible, the entire caraway oil represents a stabilizing matrix under exclusion of light and oxygen. The storage of pure terpenes, especially limonene, markedly affected their quality. Furthermore, the formation of certain minor oxidation products may be used as marker substances to recognize signs of aging in caraway essential oils as shown by both GC and HPLC analyses. In addition, HPLC analysis was well suitable for essential oil analysis, especially for the detection of more polar oxidation products such as epoxides and alcohols. Applying both methods, the detection of four novel compounds, that have been described for

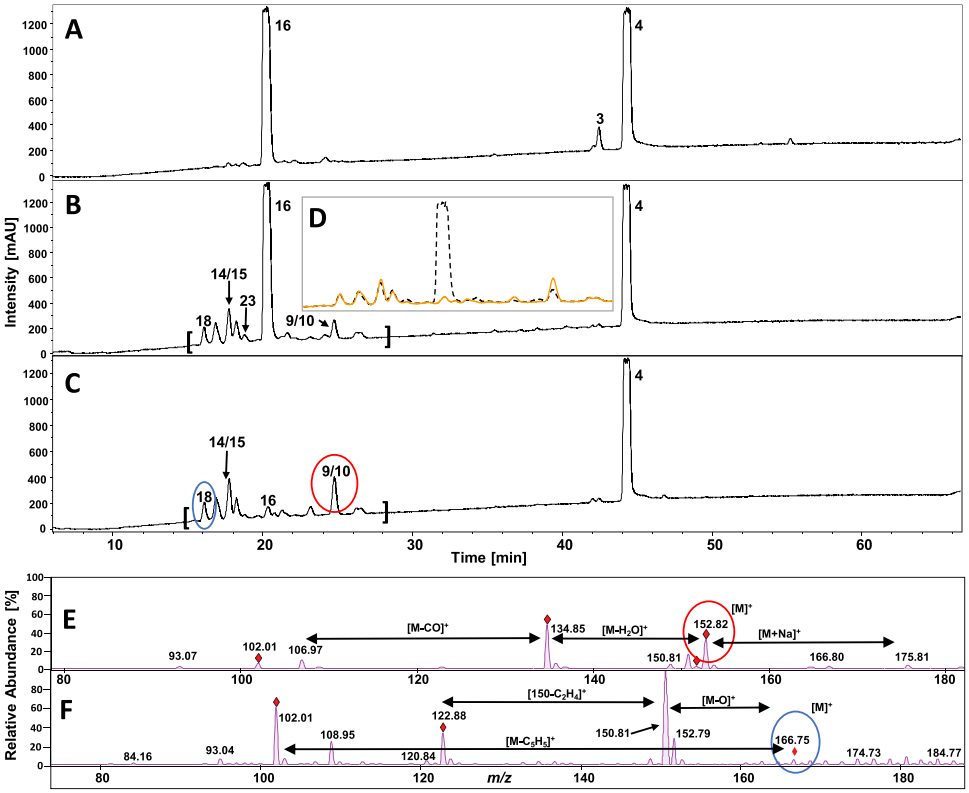

Fig. 7. UV Chromatogram (198-202 nm) of fresh caraway seeds essential oil ***D*** (**A**) and of caraway essential oil (**B**) and limonene (**C**) both stored at 40 °C for 15 months in the dark. Illustration **D** shows an overlay of the fingerprint region between 14 min and 28 min of **B (–)** and **C (-)**. LC/MS spectrum of limonene-1,2-epoxide (**E**) and carvone-1,2-epoxide (**F**). Molecular ion peaks are marked with a circle. Peak assignment according to Table 1.

caraway for the first time, was possible.

CRediT authorship contribution statement

Hannes Bitterling: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Peter Lorenz:** Validation, Writing - review & editing. **Walter Vetter:** Writing - review & editing. **Dietmar R. Kammerer:** Resources, Writing - review & editing. **Florian C. Stintzing:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

None

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4.3 Paper 3

Photo-protective effects of selected furocoumarins on *β*-pinene, *R*-(+)-limonene and *γ*-terpinene upon UV-A irradiation

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Photo-protective effects of selected furocoumarins on β-pinene, *R*-(+)-limonene and γ-terpinene upon UV-A irradiation

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ABSTRACT

The effect of furocoumarins on terpene photo-oxidation under UV-A light was investigated. For this purpose, four furocoumarins (8-methoxypsoralen, bergapten, bergaptol, bergamottin) each at a level of 5%, was added to solutions of the terpenes β-pinene, *R*-(+)-limonene and γ-terpinene in ethanol followed by UV irradiation at 366 nm. Bergaptol and bergamottin were synthesized and fully elucidated by NMR spectroscopy. UV-induced transformation of all terpenes was substantially reduced in the presence of furocoumarins. Best photo-protection was observed for γ-terpinene with the addition of bergaptol, i.e. degradation was reduced by 73% compared to the neat substance over a time period of ten days. Bergamottin (50.2%), bergapten (39.8%) and 8-methoxypsoralen (39.6%) also reduced degradation substantially. The protective effect of bergaptol was even noticeable at low concentrations of 0.1%. The main oxidation product of γ-terpinene was *p*-cymene. Limonene and β-pinene showed a predominated hydroperoxide formation under UV light which could almost completely prevented in the presence of furocoumarins. The protective effect of furocoumarins was presumably due to energy dissipation as a result of the conversion of high energetic radiation (UV-A) into visible light via fluorescence. Phosphorescence or self-quenching within the triplet state were further mechanisms avoiding reactions of excited furocoumarins with the terpenes under investigation. Hence, the photo-stability of the furocoumarins themselves correlated with the extent of their protective effect on all three terpenes.

1. Introduction

Furocoumarins are a class of secondary metabolites found in various plant species world-wide [1,2]. High furocoumarin contents have been found in the *Apiaceae* family such as celery, parsnip, and parsley or in the *Rutaceae* including all citrus fruits [2]. Furocoumarin biosynthesis is based on the fusion of a furan ring with the ubiquitous coumarin derivative umbelliferone (7-hydroxycoumarin) [3]. Structurally, linear psoralens (furan moiety fused at C6 and C7, respectively) and non-linear angelicins (furan moiety fused at C7 and C8, respectively) may be differentiated. Purposefully, furocoumarins protect plants from attacks by fungi, bacteria, and herbivores [1]. In 1834, the first furocoumarin was isolated from bergamot oil but the chemical structure remained unknown for a century. Yet, in 1933, Späth and Holzen succeeded in the structure elucidation of 8-methoxypsoralen and its chemical synthesis [4].

Medical uses of furocoumarin containing plants were already documented since ancient times by the Egyptians [5]. Nowadays, they are used in phototherapy to treat skin diseases such as vitiligo or psoriasis due to their photo-reactivity under UV light irradiation at 320–380 nm [4,6]. However, skin contact with furocoumarins in the presence of UV light can lead to sunburn-like skin injuries with heavy blisters and edema (photodermatitis) due to their phototoxic properties [1,7]. Also, photo-activated furocoumarins may form reactive oxygen species (ROS) that may penetrate epidermal and dermal endothelial cells and damage organelles and membranes. Activated furocoumarins may also directly interact with DNA to yield mono-adducts with thymine, hence their carcinogenic potential [1]. However, it has to be mentioned that furocoumarins should be considered individually, since their phototoxic potential may vary or even was found to be non-existent, such as reported for bergaptol, bergamottin and 8-geranoxypsoralen [4,8].

Photo-activation of furocoumarins takes place via light absorption by valence electrons in bonding orbitals (π orbitals) in the UV-A range

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between 320 and 380 nm [4,9]. As a result, excited electrons move from the ground state to an unoccupied orbital with unchanged electron spin, the so-called singlet state. This short-lived state is either deactivated back to the ground state by light emission (fluorescence after inner conversion (IC) without irradiation) or by intersystem crossing (ISC) (Fig. 1). ISC is linked with spin reversal (change in the spin multiplicity) which leads to the triplet state that lasts for more than one second until deactivation by phosphorescence [4,10]. In this excited triplet state, the molecule may react with various substances (e.g., DNA). Similarly, as shown for 8-methoxypsoralen (8-MOP) and coumarin, dimers of furocoumarins may be formed [4,11] or intramolecular cyclization may take place as was described for bergamottin containing a long geranyl side chain [5]. These reactions demonstrate that furocoumarins are chemically inactive without activation by light [4]. Noteworthy, the electron-resonance is strongly dependent on the solvent in which furocoumarins are dissolved [9,12]. For instance, a strong mode of action was observed in ethanol and ethanol–water mixtures [13]. Furthermore, for several furocoumarins Pathak *et al.* (1961) showed that phosphorescence occurred at higher wavelengths than fluorescence [13]. As an example, 8-methoxypsoralen activated at 360 nm emitted a yellow fluorescence (470 nm) and a yellow-green (500 nm) phosphorescence [13]. Due to these shifts to lower energy, furocoumarins could protect oxidation-sensitive substances such as terpenes from high-energy UV radiation by shielding most of the incident light.

The aim of the present investigation was to study a possible positive impact of furocoumarins on the stability of terpenes upon UV light irradiation. Namely, three terpenes (β-pinene, *R*-(+)-limonene and γ-terpinene) were UV irradiated at 366 nm for ten days without or in the presence of the four furocoumarins 8-methoxypsoralen (8-MOP), bergapten (5-MOP), bergaptol, and bergamottin (5-GOP) (Fig. 2). The latter two furocoumarins were synthesized for this purpose. Terpenes and furocoumarins were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) and quantitative gas chromatography with flame ionization detection (GC/FID), as well as by thin layer chromatography (TLC).

2. Materials and methods

2.1. Chemicals

R-(+)-limonene (limonene), (Merck, Darmstadt, Germany); boron tribromide (1 M in dichloromethane), *trans*-limonene-1,2-epoxide, *S*-(+)-carvone (Sigma Aldrich, Steinheim, Germany); 8-methoxypsoralen (xanthotoxin, 8-MOP), bergapten (5-MOP) (chemPUR, Karlsruhe, Germany); geranyl bromide (96%), *cis*-carveol (Alfa Aesar-Thermo Fischer, Kandel, Germany); α-pinene, β-pinene, γ-terpinene, *p*-cymene (Carl Roth, Karlsruhe, Germany); 1-pentanol (HPC Standards, Cunnersdorf, Germany) were used for synthesis and GC investigations. Lim-4-OOH ((2*S*,4*R*)-*p*-Mentha-6,8-diene-2-hydroperoxide; **21**) was synthesized as described in our previous investigation [15]. Na_2SO_4, NaCl, K_2CO_3, silica gel 60 (0.063 – 0.200 mm), and all solvents used were either of analytical or HPLC grade and purchased from Sigma Aldrich, Merck and Th. Geyer (Renningen, Germany). Diethyl ether (Et_2O) was distilled using a vacuum rotary evaporator (600 mbar, 38 °C) before use to remove the stabilization agent butylated hydroxytoluene (BHT). Deionized water was prepared with an Elga Purelab Classic Ultrapure Water System (Elga Labwater, Celle, Germany).

2.2. Accelerated photo-oxidation by UV light irradiation at 366 nm

Samples for photo-oxidation experiments were prepared as follows: 1.6 g of the terpene (β-pinene, γ-terpinene and limonene) was dissolved in 20 mL of ethanol (100%) in 50 mL clear glass round bottom flasks. For the samples containing furocoumarins, 80 mg (5%, w/w) of 8-methoxypsoralen, bergapten, bergaptol, and bergamottin were added to the ethanolic terpene solutions. The samples were placed in a Desaga CabUV–vis chamber (Desaga, Wiesloch, Germany) and irradiated with a UV-A lamp F8T5BLB 8 W (Sankydo Denki, Hiratsuka, Japan) at a maximum of 366 nm (hereinafter referred to as UV light). For each sample, aliquots of 500 µL were taken from the solution after 0–4, 6,7 and 10 days and mixed with 500 µL of methyl *tert*-butyl ether (MTBE) for monitoring changes by GC analysis. The samples were stored at −35 °C prior to analysis. For comparison, the initial amounts of the terpenes were referred to as 100% because their purity was inconsistent, ranging from 96% to 98%. In addition, concentration dependent experiments were performed using γ-terpinene spiked with bergaptol in concentrations of 0.1%, 1.0% and 10.0% (w/w), respectively. Furthermore, samples containing pure furocoumarins (100 mg in 20 mL ethanol) were stored under identical conditions (366 nm) for a period of 20 days.

2.3. Synthesis of bergaptol and bergamottin

Bergamottin (5-geranoxypsoralen, 5-GOP; **24**) was synthesized in two steps from bergapten (**23**) via bergaptol (**3**) according to Row *et al.* [16].

Synthesis of bergaptol (**3**): a solution of 1.0 g (4.63 mmol) of bergapten (**23**) in 50 mL of dichloromethane (DCM) was prepared and flushed with N_2. Subsequently, the reaction mixture was treated dropwise with 20.0

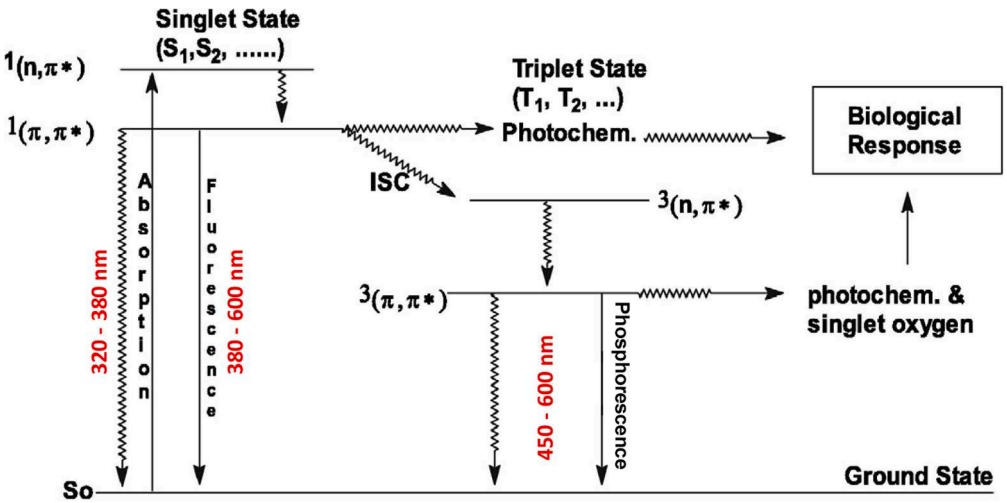

Fig. 1. Jablonski diagram representing the energy levels and intersystem crossing (ISC) of psoralens. Absorption and emission wavelengths are marked in red. Illustration modified according to Khayyat *et al.* [12,14].

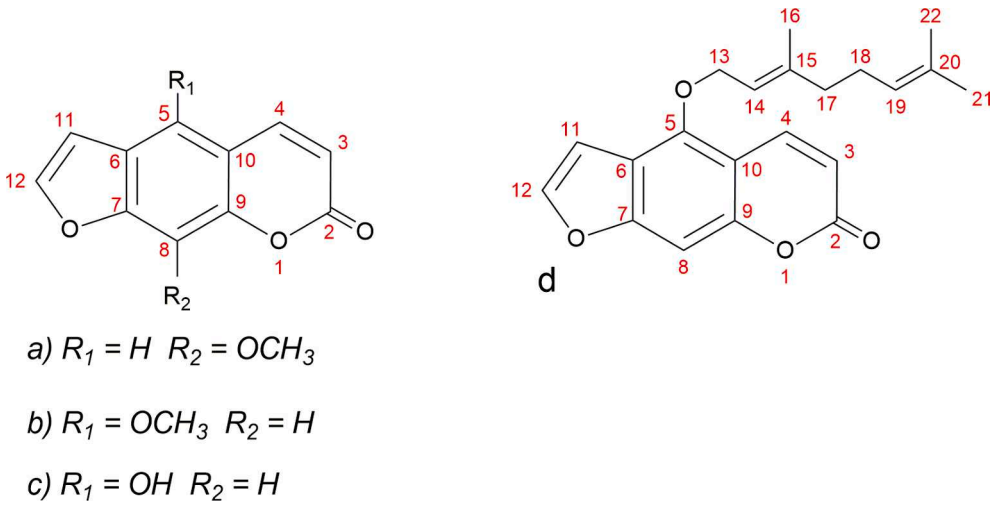

Fig. 2. Chemical structures of 8-methoxypsoralen (*a*), bergapten (*b*), bergaptol (*c*), and bergamottin (*d*) used for photo-experiments. Atom labelling of *c* and *d* according to NMR data illustrated in Table 1.

mL (20 mmol) boron tribromide (BBr_3, 1 M in DCM). Thereafter, the solution was stirred at room temperature under a gentle stream of N_2. After 3 h the reaction mixture was slowly poured into a solution of saturated $NaHCO_3$ (200 mL) resulting in the precipitation of a grey-white solid. The reaction mixture was extracted thrice with ethyl acetate (EtOAc, 3 × 150 mL). The combined EtOAc fraction was washed with 100 mL water (3x) and dried over Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was dissolved in 20 mL of ethanol and added dropwise to 250 mL *n*-hexane. A white precipitate was formed and filtered off (1.2 µm, glass microfiber), washed with *n*-hexane and dried at 60 °C in a heat cabinet. The crude product (789 mg, 3.9 mmol, 84% yield) was obtained with a purity of ≥ 99% (GC/MS). GC/MS (EI) *m/z* (%): 202 (M^+, 100), 173 (70), 117 (29), 88 (31), 62 (16).

Synthesis of bergamottin (**24**): for this purpose, 708 mg (3.5 mmol) of bergaptol (**3**) (see previous step) was dissolved in 100 mL of acetone and 800 mg (5.79 mmol) of potassium carbonate was added. To the reaction mixture 1.0 g of geranyl bromide (916 µL, 4.61 mmol) was added dropwise and heated under reflux for 1.5 h. The pH was adjusted to 6 by the addition of an aqueous citric acid solution (5%, *w/w*) followed by extraction with diethyl ether (Et_2O, 4 × 80 mL). The combined organic layer was again washed with 2 × 100 mL of H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. The resulting yellow–brown oily liquid was purified by column chromatography on silica gel (30 g). Gradient elution was started with 100% *n*-hexane, followed by addition of EtOAc to a final ration of 4:1 (*v/v*) EtOAc in *n*-hexane. The fractions obtained were identified by fluorescence upon excitation, combined and evaporated *in vacuo*. GC/MS analysis revealed the obtained product as bergamottin (**24**) with a purity of ≥ 90% (0.782 g yield, 66% of the theory). An aliquot of 270 mg was purified by centrifugally accelerated thin layer chromatography (CTLC, Chromatotron, T-Squared Technology, San Bruno, CA, USA) with a 2 mm layer (SiO_2/gypsum/fluorescence indicator 254 nm, 45:18:1.2 (*w/w/w*)), applying an *n*-hexane/EtOAc gradient from 100:0 to 50:50 (*v/v*). Pure bergamottin (31.3 mg) was obtained from the corresponding fractions after solvent removal and used for high performance liquid chromatography interfaced to high resolution mass spectrometry (HPLC-HRMS) and nuclear magnetic resonance (NMR) spectroscopy (Table 1). HPLC-APCI$^+$-HRMS *m/z* (%): 339.159 (M^+, 19); 217.049 (7); 203.034 (100); 81.071 (9).

Table 1
^{1}H- and ^{13}C- NMR data for compounds **3** and **24** (600 MHz for ^{1}H, 150 MHz for ^{13}C in DMSO-d_6).

Position	**Bergaptol**	**(3)**	Lit. [17] $CDCl_3$	**Bergamottin**	**(24)**
Carbon	^{13}C NMR δ_C (mult.)	^{1}H NMR δ_H [ΣH, mult., *J* (Hz)]		^{13}C NMR δ_C (mult.)	^{1}H NMR δ_H [mult., *J* (Hz)]
2	160.48	–	161.5	160.13	–
3	110.93	6.24 (1H, *d*, 9.7)	112.5	112.43	6.32 (1H, *d*, 9.8)
4	139.86	8.24 (1H, *d*, 9.7)	139.7	139.68	8.16 (1H, *d*, 9.8)
5	148.00	–	149.0	148.53	–
6	112.52	–	118.9	119.18	–
7	157.07	–	158.2	157.47	–
8	90.97	7.14 (1H, *s*)	94.2	93.70	7.37 (1H, *s*)
9	152.69	–	152.7	152.04	–
10	103.76	–	107.5	106.99	–
11	104.73	7.18 (1H, *dd*, 2.3, 0.9)	105.2	105.48	7.33 (1H, *dd*, 2.3, 0.6)
12	144.99	7.90 (1H, *d*, 2.3)	145.0	146.13	8.04 (1H, *d*, 2.3)
13	–	–	69.8	69.24	5.00 (2H, *br s*)
14	–	–	114.3	114.11	5.50 (1H, t, *6.8*)
15	–	–	143.1	142.28	–
16	–	–	16.8	16.33	1.63 (3H, *s*)
17	–	–	39.6	38.89	2.00 (2H, *br s*, overlapped)
18	–	–	26.3	25.67	2.00 (2H, *br s*, overlapped)
19	–	–	123.6	123.62	4.99 (1H, *br s*)
20	–	–	132.1	131.03	–
21	–	–	17.8	17.52	1.52 (3H, *s*)
22	–	–	25.8	25.40	1.59 (3H, *s*)

2.4. Analytical characterization

2.4.1. Gas chromatography (GC/MS and GC/FID)

GC measurements were performed with a Perkin Elmer Clarus 500 gas chromatograph equipped with two split/splitless injectors coupled to a Perkin Elmer Clarus 500 Mass Selective Detector with a quadrupole mass filter and a flame ionization detector (FID), respectively. Data acquisition and control of the system was achieved with TurboMass software, version 6.1.2 (Perkin Elmer) and TotalChrom version 6.3.2 for MS and FID analysis, respectively. Injection volume was 1.0 µL with a split ratio of 1:30 for samples in ethanol/methyl *tert*-butyl ether (MTBE) (50/50, *v/v*). Helium was used as a carrier gas at a constant flow of 1.0 mL/min. The analytical columns used for MS and FID analysis were

coated with 5% phenyl 95% methyl polysiloxane (60 m × 0.25 mm i.d., 0.25 µm d_f, ZB-5 ms, Phenomenex, Torrance, USA).

The following temperature program was used for both MS and FID analyses: initial temperature 60 °C, isothermal hold for 4 min, then raise to 240 °C at 6 °C/min followed by a ramp of 8 °C/min to 280 °C which was held for 8 min. The total run time was 47 min. Source and transfer line temperatures were set to 180 and 220 °C, respectively. Mass spectra were recorded in the electron ionization (EI) mode over a scan range of *m/z* 30–400. Data evaluation of the chromatograms was done with TurboMass Data Analysis Application and TotalChrome workstation (Perkin Elmer). 1-Pentanol was used as internal standard to monitor sample preparation and system accuracy. Compound assignment was based on mass spectra and linear retention indices (LRIs) according to van den Dool and Kratz (1963) as found in the NIST database as well as by using an authentic reference standard if available [18].

2.4.2. Thin layer chromatography (TLC)

All TLC experiments were conducted using 20 × 20 cm high performance thin layer chromatography (HPTLC) silica gel 60 plates (Merck, Darmstadt, Germany) containing a fluorescence indicator with an extinction wavelength of 254 nm. The samples were dissolved at a concentration level of 1 mg/mL furocoumarin in ethyl acetate and applied on silica plates using a semi-automated spray application Camag Linomat 5 (Camag, Muttenz, Switzerland). Development was achieved using a mixture of EtOAc/hexane/acetic acid (14:5.8:0.4 mL) for 30 min. Evaluation was performed at 254 nm and 366 nm using a Desaga CabUV–Vis chamber combined with a digital camera (Allied Vison Technologies, Stadtroda, Germany) and Desaga ProViDoc V5 Software (Desaga, Wiesloch, Germany).

2.4.3. LC-APCI-HRMS analysis

For the determination of the mass spectrum and exact molecular weight of bergamottin, a solution (4.8 mg/mL in MeOH) was applied to an Agilent 1290 UHPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany). Chromatographic separation of the analytes was performed on a Zorbax Eclipse Plus, RP-C_{18} column (95 Å pore size, 1.8 µm particle size, 50 × 2.1 mm i.d., Agilent Technologies, Palo Alto, CA, USA) at 40 °C. The mobile phase consisted of H_2O/HCOOH 99.8:0.2 (*v/v*; eluent A) and MeOH/HCOOH 99.8:0.2 (*v/v*; eluent B). The injection volume of each sample was 1 µL, and the gradient used was as follows: 0–29 min, 15–100% B; 29–32 min, 100% B; 32–34 min, 100–15% B; 34–36 min, 100% B, at a flow rate of 0.3 mL/min. Full scan APCI mass spectra (mass range *m/z* 150–800) of HPLC eluates were recorded during chromatographic separation in the positive/negative ionization mode.

2.4.4. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded in dimethyl sulfoxide (DMSO) at 600 (^{1}H) and 150 MHz (^{13}C), respectively, using a 600 MHz Bruker Avance III HD NMR spectrometer. Chemical shifts are reported in δ [ppm] and refer to residual solvent signals of DMSO-d_6 (^{1}H: 2.50 ppm; ^{13}C: 39.50 ppm). NMR spectra were evaluated by means of SpinWorks 3.1.7 software (Copyright 2010, K. Marat, University of Manitoba, Winnipeg, MB, Canada).

3. Results and discussion

3.1. Photo-oxidation of β-pinene by UV-A light

UV light irradiation at 366 nm caused a fast degradation of β-pinene as illustrated over a time range of ten days in Fig. 3. The relative loss (↓) of pure β-pinene was 9.4% within ten days. Compared to that, addition of furocoumarins strongly delayed the degradation of β-pinene. The protective effect of furocoumarins on photo-oxidation of β-pinene was clearly visible by the addition of bergamottin (↓ 4.4%) and bergapten

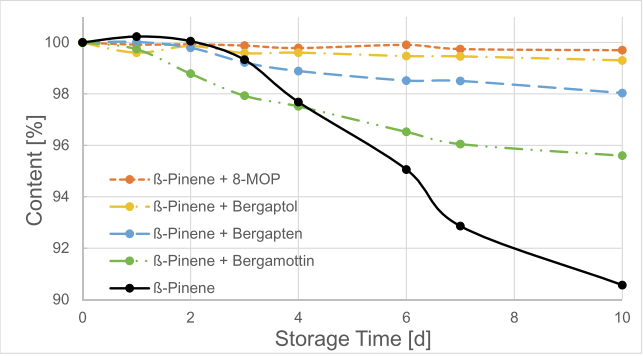

Fig. 3. Quantitative analysis (% of total GC/FID peak area, n = 2) of β-pinene samples stored for 10 days under UV light at 366 nm. Starting concentration is normalized to 100%. Note that the scale on the y-axis is from 90 to 100%.

(↓ 2.0%). An even lower degradation was observed in the presence of bergaptol (↓ 0.7%) and 8-MOP (↓ 0.3%) (Fig. 3). In agreement with that, almost no additional peaks were detected by GC/FID in the β-pinene samples containing bergaptol (Fig. 4B) and 8-MOP (Fig. 4C) after ten days upon UV-A light irradiation compared to the starting sample (Fig. 4A). These examples were selected because of the good visibility of all terpenes and furocoumarins over a period of 10–40 min (Fig. 4). In contrast, the unprotected pure β-pinene sample (Fig. 4D, no furocoumarins) featured several new minor compounds at higher GC retention times (17–27 min). These may be related to the four different oxidation sites described by Neuenschwander *et al.* (2011) in relation to the double bond in the bicyclic hydrocarbon [19]. GC/MS analysis of the irradiated β-pinene sample (ten days, no furocoumarins) revealed the formation of two β-pinene-hydroperoxides (**7**/**8**) as main photo-oxidation products (Fig. 4; Table 2). This finding was in good accordance with previous literature reports where a fast oxidation of β-pinene has frequently been reported resulting in different oxidation products upon thermal autoxidation, ozonolysis and photo-oxidation [19–21]. Also, formation of pinocarveol (**5**) and myrtenol (**6**) was confirmed by GC/MS (Table 2). The virtual absence of these degradation products in the β-pinene sample protected with furocoumarins underlined their beneficial role. Hence, effects of furocoumarins on limonene protection from UV-light were investigated next.

3.2. Photo-oxidation of R-(+)-limonene by UV-A light

Limonene, is known to be strongly affected by thermal and light induced degradation processes. Various oxidation products, including hydroperoxides, alcohols and ketones, were detected during the oxidation of limonene [15,26]. Also in the present study, a linear loss of pure limonene was observed within short time (Fig. 5), similarly to a previous real-time storage study of limonene at 25 °C and 40 °C under light exclusion [27]. After a time period of ten days under UV light the relative concentration of limonene declined (↓) by ~ 1/6th to 83.1% of the amount present at the start. Yet, the addition of furocoumarins strongly decreased the loss of limonene to less than 6% (Fig. 5). The most effective representatives, bergamottin (↓ 1.8%), 8-MOP (↓ 1.3%) and bergaptol (↓ 0.1%) almost completely prevented the deterioration of limonene (Fig. 5). For bergapten (↓ 5.9%) this effect was also visible but slightly reduced. Consequently, primary oxidation products (i.e. hydroperoxides) were virtually absent in the presence of 8-MOP (Fig. 6A). Only 0.1% Lim-4-OOH (**21**) was observed with 8-MOP addition compared to 1.4% of **21** with pure limonene (Fig. 6A). Such a protective effect of furocoumarins was also monitored for Lim-1-OOH (**18**) and Lim-5-OOH (**22**) (Fig. 6A). A comparable protective effect was also obtained in samples supplemented with bergaptol (Fig. 6B). The maximum hydroperoxide concentration was found for **18** with only 0.1% whereas all other hydroperoxide concentrations were lower. The

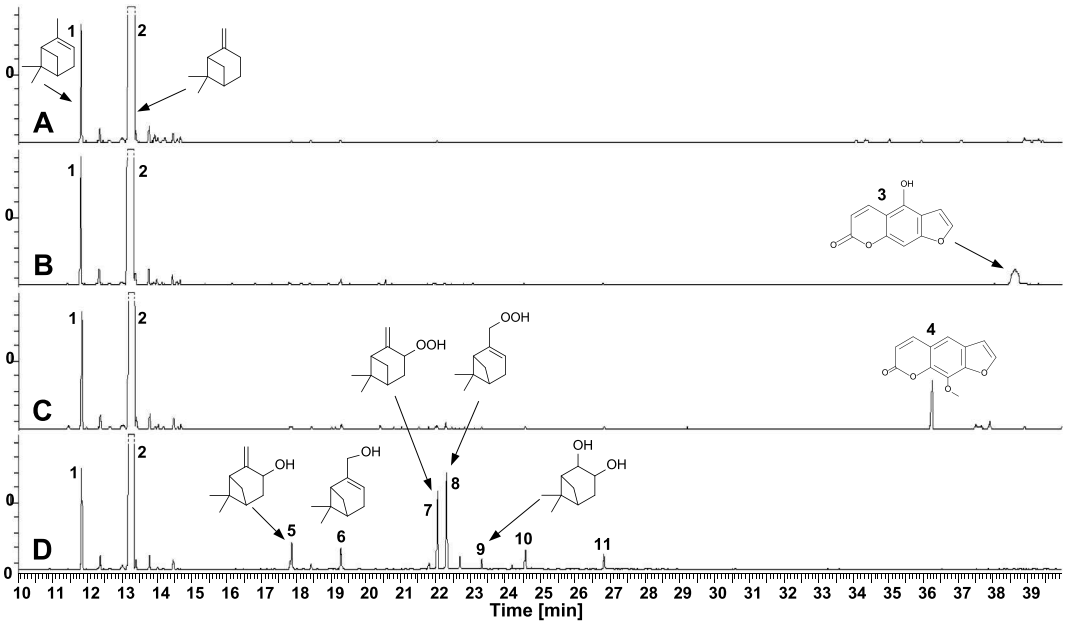

Fig. 4. GC/FID chromatograms of fresh *β*-pinene (t0, A), *β*-pinene after 10 days of irradiation at 366 nm with addition of 5% bergaptol (B) and 5% 8-MOP (C). D illustrates *β*-pinene after 10 days under UV-A light irradiation without the addition of furocoumarins. Peak assignment according to Table 2. (Data for bergapten and bergamottin are not shown).

Table 2
Compounds detected by GC/MS analysis in samples of *β*-pinene, *R*-(+)-limonene and *γ*-terpinene with furocoumarins added after photo-oxidation experiments.

No.	Compound	RI_a	RI_b	Identification	Oxidation product of	Reference
1	*α*-Pinene	937	934	RI, GC/MS*		[22]
2	*β*-Pinene	973	975	RI, GC/MS*		[22]
3	Bergaptol	2204		GC/MS*		
4	8-MOP	2043	2040	RI, GC/MS*		[23]
5	*trans*-Pinocarveol	1147	1138	RI, GC/MS	*β*-Pinene	[24]
6	Myrtenol	1200	1198	RI, GC/MS	*β*-Pinene	[22]
7	Pinene-2-hydroperoxide	1316		GC/MS^{Δ}	*β*-Pinene	
8	Pinene-1-hydroperoxide	1327		GC/MS^{Δ}	*β*-Pinene	
9	2,3-Pinanediol	1373	1319	GC/MS^{Δ}	*β*-Pinene	[25]
10	n.i.; *m/z*: 169 (M^+)	1432			*β*-Pinene	
11	n.i.; *m/z*: 152 (M^+)	1541			*β*-Pinene	
12	*p*-Cymene	1024	1025	RI, GC/MS*	*γ*-Terpinene	[22]
13	*γ*-Terpinene	1057	1060	RI, GC/MS*		[22]
14	*R*-(+)-Limonene	1034	1033	RI, GC/MS*		[22]
15	*trans*-Limonene-1,2-epoxide	1137	1141	RI, GC/MS*	*R*-(+)-Limonene	[22]
16	*cis*-Carveol	1220	1226	RI, GC/MS*	*R*-(+)-Limonene	[22]
17	*S*-(+)-Carvone	1248	1240	RI, GC/MS*	*R*-(+)-Limonene	[22]
18	(1*S*,4*R*)-*p*-Mentha-2,8-diene 1-hydroperoxide (Lim-1-OOH)	1308	1308	RI, GC/MS	*R*-(+)-Limonene	[15]
19	(1*R*,4*R*)-*p*-Mentha-2,8-diene-1-hydroperoxide (Lim-2-OOH)	1322	1321	RI, GC/MS	*R*-(+)-Limonene	[15]
20	(2*S*,4*R*)-*p*-Mentha-[1(7),8]-diene-2-hydroperoxide (Lim-3-OOH)	1333	1332	RI, GC/MS	*R*-(+)-Limonene	[15]
21	(2*S*,4*R*)-*p*-Mentha-6,8-diene-2-hydroperoxide (Lim-4-OOH)	1359	1357	RI, GC/MS*	*R*-(+)-Limonene	[15]
22	(2*R*,4*R*)-*p*-Mentha-[1(7),8]-diene-2-hydroperoxide (Lim-5-OOH)	1376	1375	RI, GC/MS	*R*-(+)-Limonene	[15]
23	Bergapten	2049	2062	RI, GC/MS*		[23]
24	Bergamottin	3647	[x]	GC/MS*		[23]

RI_a, retention indices relative to C_8-C_{20} *n*-alkanes; RI_b, reference indices; *identified by comparison with reference standard; $^{\Delta}$tentatively assigned; n.i., not identified; [x]decomposition.

identification of hydroperoxides was based on mass spectra and for **21** a synthesized reference standard was available [15,28]. As shown in section 3.1 for *β*-pinene, the limonene sample was excellently prevented from photo-oxidation and according to GC/FID investigations (Fig. 7B/C), primary (hydroperoxides; **18**–**22**) and secondary oxidation products (alcohols, ketones; **16**, **17**) could not be detected, similarly to fresh limonene (Fig. 7A) [15,28].

3.3. Photo-oxidation of *γ*-terpinene by UV-A light

γ-Terpinene is known to be readily oxidized in the presence of aerial oxygen yielding *p*-cymene with both high selectivity and high yields [29]. Compared to the terpenes reported so far, this oxidation is not based on the incorporation of oxygen but by the conversion into an aromatic compound [29]. Hence, it was interesting to monitor if furocoumarins could act protective as well in this case. As expected, UV irradiation of pure *γ*-terpinene ethanol solutions at 366 nm caused an extremely fast transformation of the terpene (Fig. 8A) into *p*-cymene (Fig. 8B). After ten days, 83.5% of the initial *γ*-terpinene amount was degraded, which is almost one order of magnitude more than in the case of *β*-pinene (section 3.1). Accordingly, the amount of *p*-cymene increased in the same order of magnitude and no other oxidation

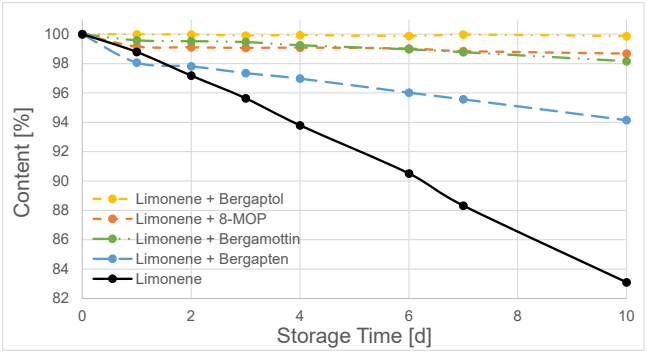

Fig. 5. Quantitative analysis (% of total GC/FID peak area, n = 2) of limonene stored for 10 days under UV light at 366 nm. Starting concentration is normalized to 100%.

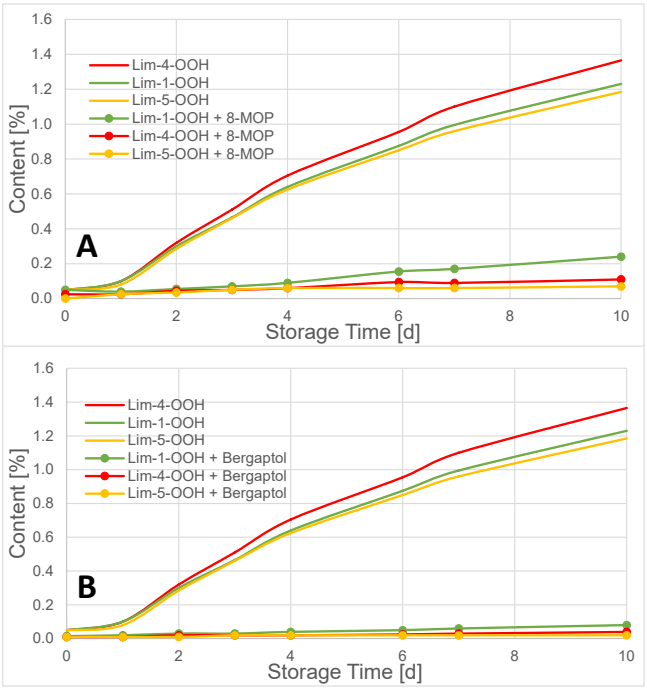

Fig. 6. Formation of limonene-hydroperoxides upon irradiation of limonene for 10 days at 366 nm in comparison to the addition of 8-MOP (A) and bergaptol (B).

products could be observed in our investigations (Fig. 8). Similarly high decomposition rates of γ-terpinene have also been described by Asikainen *et al.* (2013) during thermal oxidation experiments [29]. The fast degradation of γ-terpinene has also been described as a sign of ageing in essential lime oils [30]. Remarkably, the addition of furocoumarins also delayed the transformation of γ-terpinene (Fig. 8A). The strongest effect was achieved by adding bergaptol with an overall relative decrease of only 10.7% followed by bergamottin (↓ 33.3%) after ten days. The effect of 8-MOP (↓ 43.9%) and bergapten (↓ 43.7%) was also clearly noticeable compared to the neat substance, though less pronounced (Fig. 8A). Furthermore, the furocoumarins delayed the formation of *p*-cymene in the same order as was observed for the transformation of γ-terpinene (Fig. 8B). Hence, the strong protective effect of bergaptol on the highly sensitive γ-terpinene even exceeded the effect observed with β-pinene and limonene (see above). Up to now, little information on the stabilizing effects of furocoumarins has been published. Specifically, Musajo and Rodighiero (1962) investigated the influence of photodynamic oxidation of α-terpinene to yield the 1,4-hydroperoxide-bridged ascaridole under UV light in 1% ethanolic solutions via incorporation of oxygen [31]. Various photo-active compounds such as rose bengal or chlorophyll increased the oxidation rate. Reversly, the addition of several furocoumarins reduced the amount of ascaridole compared to the control. This was most pronounced for xanthotoxol (8-hydroxpsoralen), a hydroxypsoralen very similar to bergaptol (5-hydroxypsoralen) used in our studies [31]. All in all, the photo-sensitive γ-terpinene was clearly protected against oxidation in the presence of the furocoumarins used herein.

The strong protective effect of bergaptol on the photo-sensitive γ-terpinene prompted us to investigate the concentration dependency of this terpene-furocoumarin mixture. Hence, bergaptol was added at different concentrations to a solution of γ-terpinene (section 2.2). Remarkably, already the smallest amount of 0.1% bergaptol in the γ-terpinene solution massively reduced its degradation (↓ 50.5%) compared to the neat substance (↓ 83.5%) (Fig. 9). Hence, the three-fold amount of γ-terpinene could be maintained after 10 days. The protective effect of bergaptol on γ-terpinene was further increased by the addition of 1% (↓ 37.1%) and 10% of bergaptol (↓ 12.5%). However, at a concentration of 10% bergaptol in γ-terpinene, a sedimentation was observed, and thus the maximum solubility was reached. Arguably, higher shares of furocoumarin may offer no further protection against photo-oxidation in the present study design. Maximum concentrations of furocoumarins used in our standard experiments were within the natural range of cold pressed agrumen oils. Namely, Russo *et al.* found 2% to 5% of bergamottin in bergamot and lime essential oils [32].

3.4. Principle of the photo-protective effect of furocoumarins on terpenes

UV activation of furocoumarins into a short-lived singlet state can be deactivated by fluorescence or intersystem crossing (ISC) (Fig. 1). With regard to fluorescence, the UV-A absorption by the ethanolic furocoumarin-terpene mixtures is followed by light emission (fluorescence) of the excited furocoumarins at longer wavelength in the visible range (e.g., 470 and 460 nm for 8-MOP and bergapten, respectively) [12]. This bathochromic shift (fluorescence, $\geq$ 380 nm) skips the highly energetic wavelength range of UV-A light and is connected with lower energy levels which themselves appear to be incapable of initiating terpene oxidation [13]. The homogenous distribution of furocoumarins in ethanolic terpene solutions can therefore act as a protective shield against high energy radiation. This is also supported by the strikingly high molar extinction coefficients of furocoumarins, i.e. 3.03×10^4 L $\times$ mol^{-1} $\times$ cm^{-1} for 8-MOP [33].

The second process, ISC, leads to furocoumarins activated to the triplet state in which they can react with various compounds. On the one hand, this reactive state is responsible for the phototoxicity of some basic furocoumarins such as psoralen and bergapten [4]. On the other hand, additional electron withdrawing groups, such as hydroxyl functions, alter the resonance of the molecule which removes the photosensitizing properties of the furocoumarin as was exemplarily shown for bergaptol and xanthotoxol [4,31].

Last but not least, molecules in triplet state predominantly react with other molecules in triplet state which are not found in the case of terpenes. Hence, without a direct reaction partner, electrons in the triplet state are deactivated via phosphorescence, i.e. without affecting terpenes. Hereby, phosphorescence light emissions occur at even longer wavelengths than fluorescence and a bathochromic shift up to 500 nm could be detected [9,13]. Compared to the high-energetic UV-A light, the low energy in the visible range seems to have no impact on terpene oxidation. Apart from phosphorescence and reactions with other molecules in the triplet state, furocoumarins in their triplet state can also react intramolecularly by formation of dimers, which is known as self-quenching [5,11]. Hence, it was necessary also to investigate the fate of furocoumarins (section 3.5).

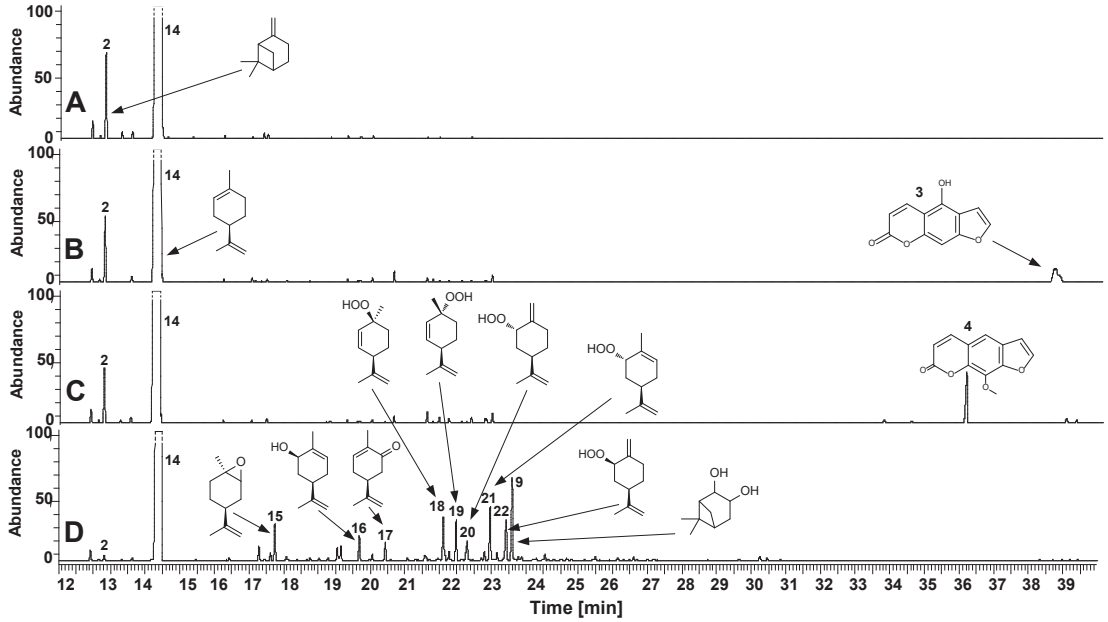

Fig. 7. GC/FID chromatograms of fresh limonene (t0, A), limonene after 10 days of irradiation at 366 nm with addition of bergaptol (B) and 8-MOP (C). D illustrates limonene after 10 days under UV-A light irradiation without furocoumarin addition. Peak assignment according to Table 2. (Data for bergapten and bergamottin are not shown).

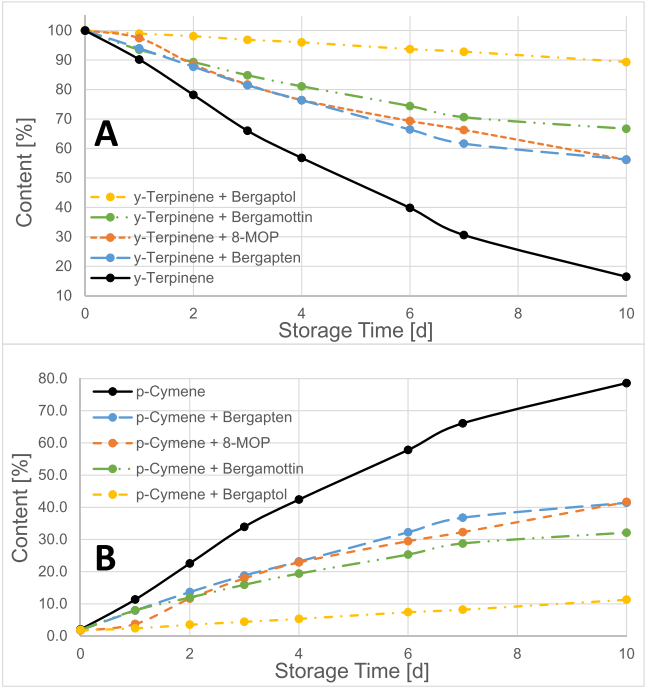

Fig. 8. Quantitative analysis (% of total GC/FID peak area, n = 2) of γ-terpinene samples stored for 10 days under UV light at 366 nm (A) and antiparallel *p*-cymene formation in γ-terpinene samples stored under identical conditions (B). Starting concentration was normalized to 100%.

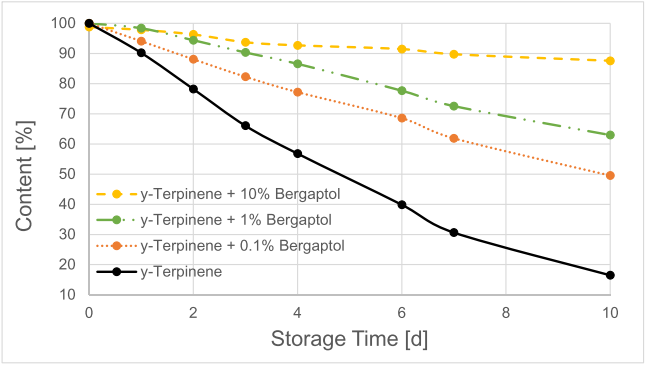

Fig. 9. Quantitative analysis (% of total GC/FID peak area, n = 2) of γ-terpinene degradation under UV light in the presence of 0.1%, 1.0% and 10% bergaptol.

3.5. *Photodegradation of furocoumarins under UV-A light irradiation*

The behavior of pure furocoumarins under UV light without terpene addition was investigated under the same conditions as described in section 2.2. All solutions that were clear and transparent in the beginning became yellow to brown after 20 days of irradiation (Fig. 10). 8-MOP, bergapten, bergaptol and bergamottin still showed fluorescence at 366 nm, which indicated that they were only partly degraded as investigated by TLC experiments (Fig. 11). In the present study TLC was used to separate irradiated furocoumarin solutions (Fig. 11). Hereby, it was possible to discern several breakdown products, that escaped detection via GC, which was most pronounced for bergapten showing several new fluorescing bands. The components on the starting front most likely represent the photo-dimers or possibly even larger polymeric compounds (Fig. 11). The formation of photo-dimers upon UV light absorption has also been described for coumarin in ethanolic solutions. In this excited triplet state coumarin directly reacts with ground state coumarin resulting in self-quenching and dimer formation [11]. Giménez-Arnau and co-workers also reported on intramolecular photocyclization of bergamottin due to the geranyl side chain and two different isomers could be isolated and characterized [5].

Table 3 lists the furocoumarin content of samples containing terpenes (section 2.2) upon ten days under UV light. Degradation was most pronounced for bergapten. In the γ-terpinene sample, only 5.7% of the initial furocoumarin was present at the end of the photo-experiments (Table 3). Lowest furocoumarin degradation was found for bergaptol. In the presence of β-pinene (86.2%), γ-terpinene (76.8%), and limonene

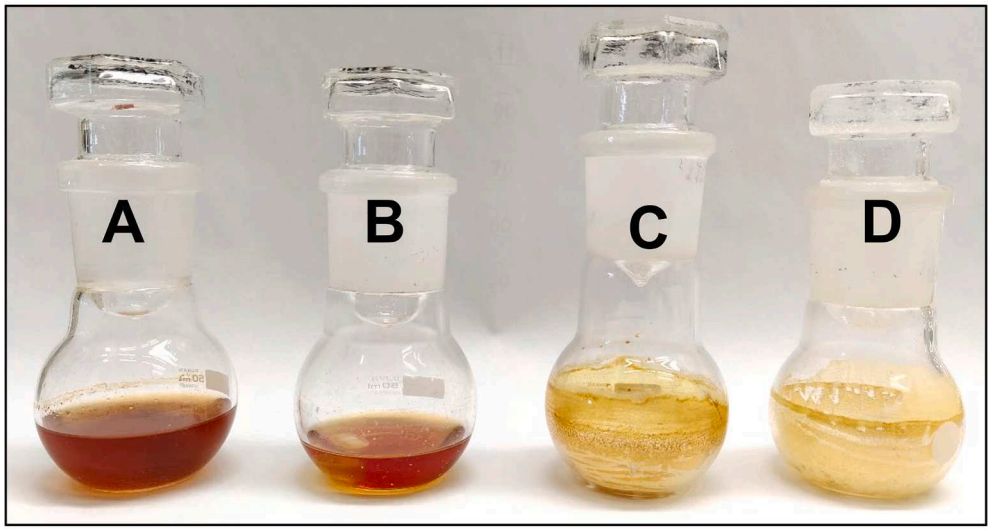

Fig. 10. Bergamottin (A, in ethanol), bergaptol (B, in ethanol), bergapten (C, after solvent removal) and 8-MOP (D, after solvent removal) illuminated for 20 days at 366 nm.

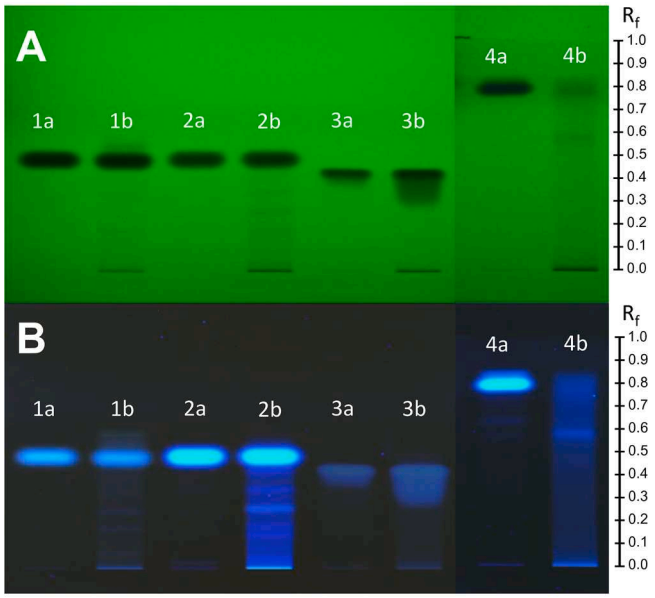

Fig. 11. Thin layer chromatography of 8-MOP (1a), bergapten (2a), bergaptol (3a) and bergamottin (4a) analyzed at 254 nm (A) and 366 nm (B). The samples b were UV-irradiated for 20 days (1b, 8-MOP; 2b, bergapten; 3b, bergaptol; 4b, bergamottin).

Table 3
Average percentage share (n = 2) of furocoumarins after ten days of irradiation (**T10**) in samples containing both furocoumarins and terpenes, prepared as described in section 2.2.

	T10 8-MOP [%]	T10 Bergapten [%]	T10 Bergaptol [%]	T10 5-GOP [%]	Range
β-Pinene	43.8	13.7	86.2	12.2	**74.0**
γ-Terpinene	36.3	5.7	76.8	13.3	**71.7**
Limonene	58.7	6.7	81.0	9.1	**74.3**
Range	**22.4**	**8.2**	**9.4**	**4.2**	

8-MOP, 8-Methoxy psoralen (Xanthotoxin); 5-GOP, 5-Geranoxpsoralen (Bergamottin).

(81.0%) of the initial furocoumarin was present after ten days. This correlated with the high photo-protective effect of this furocoumarin. The degradation of the furocoumarins used in the experimental series (section 2.2) was mainly dependent on its chemical structure, rather than on terpene addition. This finding is evidenced by the fact that the lowest range was found within bergapten (8.2) and bergamottin (4.2) samples (Table 3).

4. Conclusion

The protective effect of furocoumarins on the oxidation and alteration of terpenes upon UV-A light irradiation was clearly demonstrated by spiking *β*-pinene, *γ*-terpinene and limonene with furocoumarins. Namely, all four furocoumarins 8-MOP, bergapten, bergaptol, and bergamottin strongly reduced the degradation of all compounds. The effect was most pronounced for bergaptol, which at the same time is a non-phototoxic substance. Compared to pure terpenes, the degradation was reduced by 8.7% (*β*-pinene), 16.8% (limonene) and 72.8% (*γ*-terpinene). Furthermore, *γ*-terpinene is suggested as marker compound for photo-experiments, as this terpene is highly susceptible towards UV light irradiation and was almost completely degraded under the experimental conditions. For limonene, the formation of hydroperoxides was significantly reduced in the presence of all furocoumarins, and this effect was most pronounced with 8-MOP and bergaptol. This is an important finding since hydroperoxides may lead to allergenic skin reactions in cosmetics and care products [28]. Further investigations indicated that the protective principle is most likely based on energy dissipation due to fluorescence and phosphorescence, as well as self-quenching of furocoumarins. Samples stored under exclusion of light did not show any change over the observed time period. Hence, furocoumarins could be particularly useful for the achievement of longer storage stabilities of terpenes and essential oils where exposure to light cannot be fully excluded. For instance, the addition of selected furocoumarins to cosmetics and care products could be conceivable to reduce losses of fragrance terpenes. However, individual furocoumarin should be carefully tested in advance on possible phototoxic effects. At least in the case of bergamottin and bergaptol which were used in our investigations phototoxicity does not seem to exist [4,8]. Also, it would be worth to investigate the photo-protective effects of essential agrumen oils and other essential oils in which furocoumarins are naturally present.

CRediT authorship contribution statement

Hannes Bitterling: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Peter Lorenz:** Validation, Writing – review & editing. **Walter Vetter:** Writing – review & editing. **Dietmar R. Kammerer:** Resources, Writing – review & editing. **Florian C. Stintzing:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotochem.2021.113623.

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4.4 Paper 4

Photo-protective effects of furocoumarins on terpenes in lime, lemon and bergamot essential oils upon UV light irradiation

Hannes Bitterling, Lilo Mailänder, Walter Vetter, Dietmar Rolf Kammerer, and Florian Conrad Stintzing

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ORIGINAL PAPER

Photo-protective effects of furocoumarins on terpenes in lime, lemon and bergamot essential oils upon UV light irradiation

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Abstract

The impact of naturally occurring furocoumarins on essential agrumen oils, namely bergamot, lime and, lemon, was investigated upon exposure to UV-A light. For this purpose, the oils were initially freed from furocoumarins and coumarins by precipitation in cold hexane. Such pretreated oils, as well as samples of these oils spiked with separated furocoumarins were irradiated for up to 10 days. All essential oils devoid of furocoumarins showed a massive degradation of the predominant terpenes *R*-(+)-limonene and γ-terpinene. For lime and lemon essential oils 10% and 7.5% of the initial *R*-(+)-limonene amount was degraded within 10 days, respectively. In addition, a noticeable hydroperoxide formation was observed. For γ-terpinene, this effect was even more pronounced and in both, lime and lemon essential oil samples, the terpene was entirely converted into *p*-cymene after 6 days. In comparison, addition of 5% furocoumarins to the essential oils decelerated the photo degradation of *R*-(+)-limonene and γ-terpinene by up to one order of magnitude. The protective effect of furocoumarins, presumably due to bathochromic shifts of emitted light to less harmful longer wavelengths, also improved the olfactory quality. The results demonstrate that blends of non-volatile furocoumarins and volatile terpenes extend the shelf-life of light-sensitive agrumen oils.

Keywords Essential oil stability · Furocoumarin fractionation · Photochemistry · Photooxidation · UV-A light

Introduction

Citrus fruits are mainly produced in the sub-tropical regions and consumed worldwide [1]. The annual production volume of these important industrial crops exceeds 100 million tons [2]. They are consumed fresh, used for juice production or for the extraction of raw materials, such as pectin and essential oils [3]. During juice production, the essential citrus oils (agrumen oils) located in the peel are separated from the fruits by a process called pellatrice [4]. In this process, essential oils are recovered from the fruits by direct peel abrasion followed by centrifugation. Contrary to other plants, this process does not take advantage of the volatility of the essential oils. As a consequence, non-volatile compounds, such as coumarins, polymethoxyflavones, and furocoumarins, which are also mainly located in the peel of the fruits, are transferred into the essential oils. This non-volatile fraction can make up to 15% in cold-pressed citrus essential oils [4, 5].

Citropten, bergapten, bergaptol, bergamottin, and imperatorin are common coumarins and furocoumarins in citrus fruits [5, 6]. Highest contents were found in essential oils from bergamot (*Citrus bergamia* Risso et Poit) and Persian lime ((*Citrus* × *aurantiifolia* Christm. et Panz.) Swingle) with a total contribution of up to 5% to the essential oils [5, 7]. The biosynthesis of furocoumarins starts with phenylalanine and involves several enzymatic steps until formation of the ubiquitous coumarin precursor umbelliferone. Depending on the position of an additional furan ring, linear so-called psoralens and non-linear angelicins are formed (Fig. 1). Psoralens are the dominant group of furocoumarins in citrus fruits [3, 8]. Next to citrus fruits and other members of the *Rutaceae* family, this class of plant secondary metabolites is also found in members of the *Apiaceae* family, such as celery, parsnip, and parsley [8].

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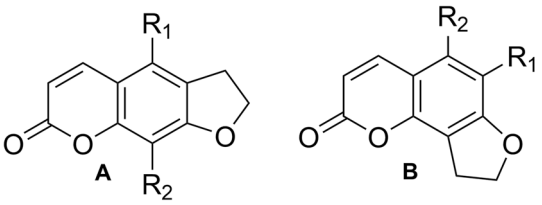

Fig. 1 Structures of psoralen (**A**) and angelicin (**B**), which are found as various derivatives in plants

Furocoumarins are produced by plants to protect them from insects, fungi, and bacteria. This is why increased accumulation was detected on infected plant parts, showing that plants directly respond to damage [9]. Highest furocoumarin concentrations were found in microbially infected parsnip with concentrations up to 3 g/kg [10]. Hence, furocoumarins have also been used as natural pesticides in agricultural industry to protect crops from microbial contamination [11]. In human health studies, furocoumarins proved to have antioxidant, anti-inflammatory, and bone health promoting properties [3]. In addition, furocoumarins have also been used for the treatment of skin diseases, such as vitiligo and psoriasis in a therapy called PUVA (psoralen + UV-A) [11]. However, in most cases, furocoumarins have been associated with negative physiological effects as they may act as photo-sensitizers. This means that contact with furocoumarins and exposure to UV light may lead to sunburn like skin injuries called phytophotodermatitis. Photo-activated furocoumarins can also directly interact with the DNA to form mono-adducts with the base thymine, thus bearing also a carcinogenic potential [11]. For this reason, there are strict regulations for the use of essential oils containing furocoumarins in cosmetics. The content of photo-sensitizers such as furocoumarins is restricted to a maximum of 1 ppm in leave-on products, which plays a decisive role especially when using natural essential agrumen oils [12]. For that reason, several essential agrumen oils are rectified by vacuum distillation or chromatography to remove furocoumarins [13]. However, it should be mentioned that several factors (specific molecular structure, concentration, irradiation wavelength, part of skin) have an impact on the photosensitizing properties of furocoumarins [14–16]. As described by Scott et al. [17] the photosensitivity is strongly affected by the presence/absence of functional groups, such as methyl or hydroxyl functions. In addition, long side chains containing alkyl groups may lead to a total loss of the photosensitizing properties [15, 17]. For this reason, careful attention should be paid to the respective furocoumarin profile. Photoactivation of furocoumarins mainly occurs at 320–380 nm, which is also the wavelength range in PUVA therapy. However, furocoumarins may also absorb these short wavelength ranges (UV-A), and releasing light of higher wavelengths via fluorescence and/or phosphorescence [18]. This process may have a beneficial effect on the shelf-life of essential oils. Irradiation in this wavelength range excites outer electrons from the ground state into an unoccupied orbital with unchanged electron spin, the so-called singlet state. This state is very short-lived and can be returned to the ground state by fluorescence. In addition, spin reversal via intersystem crossing (ISC) can lead to the triplet state, which can last for > 1 s [17, 19]. In this excited triplet state, reaction with substrate molecules (e.g., DNA) may happen. Moreover, dimer formation among furocoumarins can occur, which has been demonstrated for coumarin [20]. Notably, without activation by light, no chemical activity has been described for furocoumarins [17].

The aim of the present study was to investigate the impact of furocoumarins on terpene stability in authentic essential oils upon UV light irradiation. For this purpose, three commercial cold-pressed essential oils, namely, lemon, lime, and bergamot, were initially freed from furocoumarins. The resulting furocoumarin-free essential oils and re-spiked counterparts were used for photo-oxidation experiments. Essential oils were analyzed by gas chromatography coupled with mass spectrometry (GC/MS) and quantitative gas chromatography with flame ionization detection (GC/FID).

Materials and methods

Chemicals

The following chemicals and reference compounds were used: *R*-(+)-limonene (limonene) (Merck, Darmstadt, Germany); *β*-linalool, neryl acetate, citral (neral, geranial) (Sigma Aldrich, Steinheim, Germany); *α*-pinene, *β*-pinene, *γ*-terpinene, *p*-cymene (Carl Roth, Karlsruhe, Germany); 8-methoxypsoralen (xanthotoxin, 8-MOP), bergapten (5-MOP) (chemPUR, Karlsruhe, Germany); 1-pentanol (HPC Standards, Cunnersdorf, Germany); (2*S*,4*R*)-*p*-mentha-6,8-diene-2-hydroperoxide (Lim-2-OOH), was synthesized as shown before [21]. Na_2SO_4, NaCl, and all solvents used were either of analytical or HPLC grade and purchased from Sigma Aldrich, Merck and Th. Geyer (Renningen, Germany). Deionized water was prepared with an Elga Purelab Classic Ultrapure Water System (Elga Labwater, Celle, Germany). The following cold-pressed essential oils used were purchased from Naturamus (Aichelberg, Germany): Lime ((*Citrus* x *aurantiifolia* Christm. et Panz.) Swingle), bergamot (*Citrus bergamia* Risso et Poit), and lemon (*Citrus limon* (L.) Osbeck).

Separation of furocoumarins from essential oils

To separate the furocoumarin fraction from the original essential oil, 150 g of lemon, lime, and bergamot oil was mixed with 800 mL *n*-hexane, respectively. Subsequently, the mixture was cooled to − 80 °C for 6 h in an ultra-low temperature freezer. The precipitate thus formed was filtered off from the essential oil-hexane mixture using a 1.2 µm glass fiber filter (LLG Labware, Meckenheim, Germany) and a vacuum suction flask. The residue was again washed with cold *n*-hexane. Following this, the residue was rinsed with ethyl acetate (EtOAc). Both the hexane and EtOAc fractions were dried over NaS_2O_4 and evaporated to dryness *in vacuo*. A separation of furocoumarins from essential oils by distillation was circumvented, because exposure to heat should be avoided to exclude any thermal degradation.

Accelerated photo-oxidation by UV-A light

Samples for photo-oxidation experiments were prepared as follows: 1.6 g of the furocoumarin-depleted essential oils ("Separation of furocoumarins from essential oils" section; lime, lemon and bergamot) were dissolved in 20 mL of ethanol (100%) in 50 mL clear glass round bottom flasks. For comparison, 80 mg (5%, *w*/*w*) of the separated furocoumarin fractions were added to the ethanolic essential oil solutions. Both samples were placed in a Desaga CabUV–Vis chamber (Desaga, Wiesloch, Germany) and irradiated with a UV-A lamp (F8T5BLB 8 W, Sankydo Denki, Hiratsuka, Japan) at a maximum of 366 nm (hereinafter referred to as UV light) for 10 days. For each sample, aliquots of 500 µL were taken after 0–4, 6, 7 and 10 days and mixed with 500 µL of methyl *tert*-butyl ether (MTBE) for monitoring changes by GC analysis. The samples were stored at − 35 °C until analysis.

Analytical characterization

Gas chromatography (GC/MS and GC/FID)

GC measurements were performed with a Perkin Elmer Clarus 500 gas chromatograph equipped with two split/splitless injectors coupled to a Perkin Elmer Clarus 500 Mass Selective Detector with a quadrupole mass filter and a flame ionization detector (FID), respectively. Data acquisition and control of the system was achieved with TurboMass software, version 6.1.2 (Perkin Elmer) and TotalChrom version 6.3.2 for MS and FID analysis, respectively. Injections (1.0 µL) were performed in split mode (split ratio 1:30) for samples in ethanol/methyl *tert*-butyl ether (MTBE) (50/50, *v*/*v*). Helium was used as a carrier gas at a constant flow of 1.0 mL/min. The analytical columns used for MS and FID analysis were coated with 95% methyl 5% phenyl polysiloxane (60 m × 0.25 mm i.d., 0.25 µm d_f, ZB-5 ms, Phenomenex, Torrance, USA).

The following temperature program was used for both MS and FID analyses: initial temperature 60 °C, isothermal hold for 4 min, then raise to 240 °C at 6 °C/min followed by a ramp of 8 °C/min to 280 °C, which was held for 8 min. The total run time was 47 min. Ion source and transfer line temperatures of the MS were set to 180 and 220 °C, respectively. Mass spectra were recorded in the electron ionization (EI) mode over a scan range of *m/z* 30–400. Data evaluation of the chromatograms was performed with TurboMass Data Analysis Application and TotalChrome workstation (Perkin Elmer). Samples were analysed in duplicate and 1-pentanol was used as internal standard to monitor sample preparation and system accuracy. Compound assignment was based on mass spectra and linear retention indices (LRIs) according to van den Dool and Kratz (1963) as found in the NIST database as well as using authentic reference standards if available [22].

Spectrophotometric analysis (UV/Vis)

Solutions of the commercially available furocoumarins bergapten (5-methoxypsoralen) and xanthotoxin (8-methoxypsoralen) were prepared in acetonitrile (0.1 mmol). Acetonitrile was used because of good solubility of both furocoumarins as well as low interference in the UV wavelength range. The samples were analysed using a Lambda 2 UV/Vis double beam spectrophotometer (Perkin Elmer, Waltham, MA, USA) and 1.5 mL PMMA (poly(methyl methacrylate)) semi micro-cuvettes (Brand, Wertheim, Germany). Full scan spectra were recorded from 200 to 500 nm with a band width of 2 nm. For the determination of extinction factors, measurements were performed at 366 nm. The molar attenuation coefficient ε (a.k.a. molar extinction coefficient or factor) was calculated using the Lambert–Beer equation $\varepsilon = E/c \times d$, where E is the dimensionless extinction, c is the concentration in mol/L and d is the length of the light path (width of the cuvette) [23].

Results and discussion

Fractionation and analysis of essential agrumen oils

Due to the higher polarity, we aimed at removing furocoumarins by precipitation from hexane solutions in the cold ("Separation of furocoumarins from essential oils" section). This procedure was successful and yielded terpene fractions that were free from furocoumarins as exemplified for lime essential oil in Fig. 2A. Likewise, the precipitation of furocoumarins from the cold-pressed essential lime and

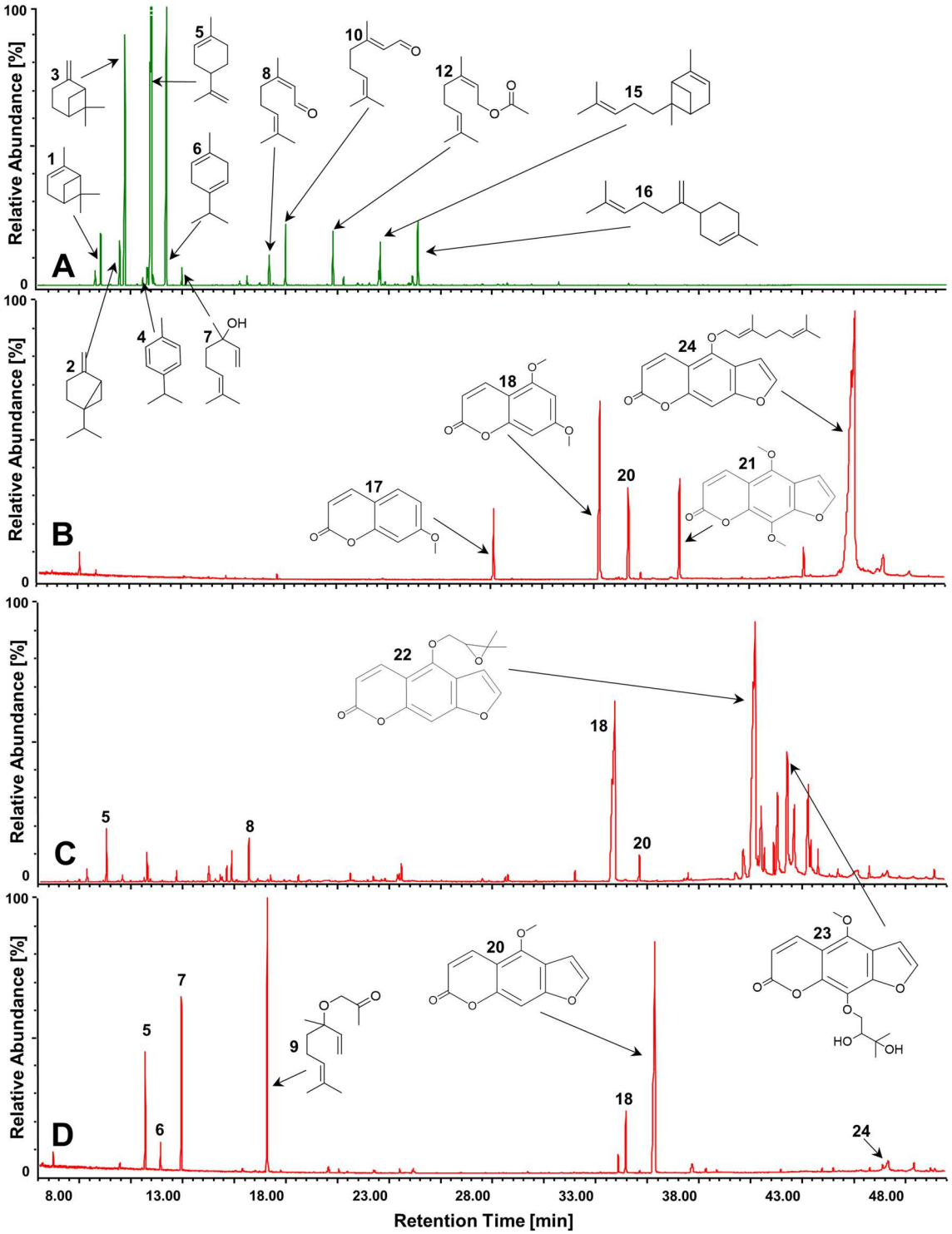

Fig. 2 GC/FID chromatograms showing the effect of furocoumarin precipitation with hexane fraction of lime essential oil (**A**) and precipitates of (**B**) lime, (**C**) lemon, and (**D**) bergamot essential oil. Peak labels refer to terpenes α-pinene (**1**), sabinene (**2**), β-pinene (**3**), *p*-cymene (**4**), *R*-(+)-limonene (**5**), γ-terpinene (**6**), β-linalool (**7**), neral (β-citral; **8**), linalyl acetate (**9**), geranial (α-citral; **10**), neryl acetate (**12**), trans-α-bergamotene (**15**), and β-bisabolene (**16**) and the coumarins and furocoumarins 7-methoxycoumarin (herniarin; **17**), citropten (**18**), bergapten (**20**), isopimpinellin (**21**), oxypeucedanin (**22**), biacangelicin (**23**) and bergamottin (**24**). See also Table 1

lemon oils worked excellently (Fig. 2B, C). The GC elution behavior of the furocoumarins was in agreement with their noticeably higher molecular mass and boiling point compared to most of the terpenes in the essential oils. The mass of the precipitated fractions was 7.3 g (4.9%) for lime, 1.9 g (1.3%) for bergamot and 1.1 g (0.7%) for lemon essential oil, respectively. The amount of furocoumarins separated from the oils was in good accordance with previous literature reports on total furocoumarin levels [5]. For lime essential oil, the five most prominent coumarins and furocoumarins were identified by GC/MS (Fig. 1B), namely, 7-methoxycoumarin (herniarin, **17**), citropten (**18**), bergapten (**20**), isopimpinellin (**21**), and bergamottin (**24**) [24]. In addition, the three most abundant coumarins and furocoumarins in lemon essential oil, namely, citropten (**18**), oxypeucedanin (**22**), and biacangelicin (**23**) were assigned by GC/MS (Fig. 1C) [25, 26]. Only some minor furocoumarins could not be identified in these two oils. For bergamot essential oil, the main substances identified in the precipitate were the coumarin citropten (**18**) and the furocoumarins bergapten (**20**), as well as bergamottin (**24**), which is in accordance with reports by Costa et al. [13]. The share of furocoumarins in the separated fractions was > 98% for lime and > 97% for lemon. Regrettably, for bergamot essential oil, the purity of the furocoumarin fraction was only 35% due to high residual contents of the terpenes *R*-(+)-limonene, linalool, and linalyl acetate (Fig. 1D). Presumably, a high proportion of linalyl acetate resulted in poorer separation of the substances. Still, this procedure brought about an enrichment of furocoumarins by more than one order of magnitude considering their initial concentrations of approx. 2% in this essential oil [27]. Moreover, the furocoumarin profile of the precipitate corresponded well with the composition in the cold-pressed essential oil (Table 1).

Effect of UV irradiation of essential oils on their *R*-(+)-limonene contents

R-(+)-Limonene is one of the most abundant monoterpenes in all three essential agrumen oils used in our study [1, 13]. GC/FID analysis confirmed initial *R*-(+)-limonene concentrations of 40.7% in bergamot, 50.6% in lime and

Table 1 Compounds detected by GC/MS in lime, lemon and bergamot essential oils

No	Compound	RI_a	RI_b	Identification	References
1	*α*-Pinene	937	936	RI, GC/MS*	[28]
2	Sabinene	973	976	RI, GC/MS	[28]
3	*β*-Pinene	982	982	RI, GC/MS*	[28]
4	*p*-Cymene	1024	1028	RI, GC/MS*	[28]
5	*R*-(+)-Limonene	1032	1034	RI, GC/MS*	[28]
6	*γ*-Terpinene	1057	1063	RI, GC/MS*	[28]
7	*β*-Linalool	1084	1102	RI, GC/MS*	[28]
8	Neral (*β*-citral)	1235	1240	RI, GC/MS*	[28]
9	Linalyl acetate	1246	1254	RI, GC/MS*	[13]
10	Geranial (*α*-citral)	1264	1272	RI, GC/MS*	[28]
11	(1*R*,4*R*)-*p*-Mentha-2,8-diene-1-hydroperoxide (Lim-1-OOH)	1322	1321	RI, GC/MS	[21]
12	Neryl acetate	1349	1360	RI, GC/MS*	[28]
13	(2*S*,4*R*)-*p*-Mentha-6,8-diene-2-hydroperoxide (Lim-2-OOH)	1359	1357	RI, GC/MS*	[21]
14	(2*R*,4*R*)-*p*-Mentha-[1(7),8]-diene-2-hydroperoxide (Lim-3-OOH)	1376	1375	RI, GC/MS	[21]
15	*trans*-*α*-Bergamotene	1440	1437	RI, GC/MS	[28]
16	*β*-Bisabolene	1515	1512	RI, GC/MS	[28]
17	7-Methoxycoumarin	1660	1732	RI, GC/MS	[24]
18	Citropten	1943	1986	RI, GC/MS	[24]
19	Xanthotoxin (8-MOP)	2043	2040	RI, GC/MS*	[24]
20	Bergapten (5-MOP)	2049	2062	RI, GC/MS*	[24]
21	Isopimpinellin	2196	2240	RI, GC/MS	[24]
22	Oxypeucedanin	2418	2483	RI, GC/MS	[24]
23	Biacangelicin	2534		GC/MS^{Δ}	
24	Bergamottin	3647	^{x}	GC/MS^{Δ}	[24]

RI_a, retention indices relative to C_8–C_{20} *n*-alkanes; RI_b, reference indices; *identified by comparison with reference standard; $^{\Delta}$tentatively assigned; ^{x}decomposition

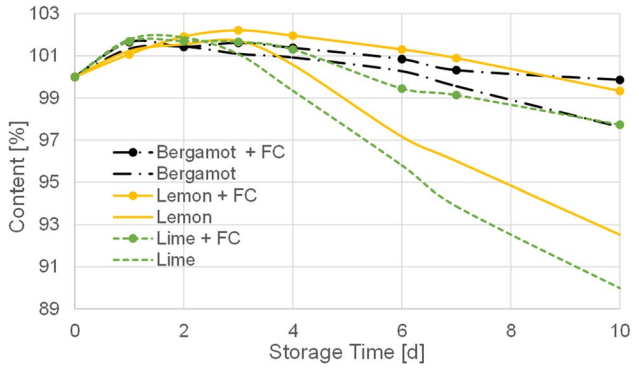

Fig. 3 Relative degradation of *R*-(+)-limonene in bergamot, lime, and lemon essential oils during 10 days of storage under UV-A light. Samples containing furocoumarins are marked with dots on the graph. For a better comparison, initial amounts were set to 100% as reference and the degradation was expressed as relative percentage changes

68.2% in lemon essential oils, respectively (data not shown). UV-A light irradiation as described in "Accelerated photooxidation by UV-A light" section caused a fast degradation of *R*-(+)-limonene, especially in the case of lime and lemon oils freed from furocoumarins according to "Separation of furocoumarins from essential oils" section (Fig. 3). Interestingly, furocoumarin addition at a level of 5% (*w*/*w*) to these essential oils devoid of furocoumarins increased the UV stability of *R*-(+)-limonene (Fig. 3). Thus, degradation (↓) of *R*-(+)-limonene in lime and lemon (10.0% and 7.5%, respectively, in the absence of furocoumarins) was reduced by a factor of 4 and 10 to 2.3% for lime and 0.7% for lemon essential oils after 10 days (Fig. 3). It is particularly noteworthy that the added amount of furocoumarins was close to the highest levels previously detected in cold-pressed essential citrus oils [5]. For bergamot essential oil, the degradation rate without furocoumarins was lower (Fig. 3). However, the protective effect of furocoumarins on *R*-(+)-limonene degradation was still larger than one order of magnitude (2.3% degradation in the absence of furocoumarins vs. 0.1% with furocoumarins). This is remarkable, because the purity of the spiked furocoumarin fraction was lower in this case.

As *R*-(+)-limonene is the key terpene in bergamot, lime, and lemon essential oils, its substantial degradation has a strong impact on the component profile and consequently most likely also on the odor of the essential oil, which is an important quality factor for its use in the flavoring and cosmetics industry. Its fruity lemon-like odor is the key impression of many citrus essential oils and *R*-(+)-limonene is further used in the flavor and fragrance industry for the synthesis of various derived flavoring compounds, such as *p*-cymene and thujaplicins [29, 30]. The formation of hydroperoxides is an additional problem of *R*-(+)- imonene degradation, that in rare cases, may lead to allergenic reactions when undiluted essential oils are applied to the skin [31]. Remarkably, formation of limonene-1-hydroperoxide (Lim-1-OOH, **11**), limonene-2-hydroperoxide (Lim-2-OOH, **13**) and limonene-3-hydroperoxide (Lim-3-OOH, **14**) was massively reduced when furocoumarins were added to the lime and lemon essential oil samples (Fig. 4). For lime essential oil, the maximum concentration of 0.6% Lim-3-OOH without furocoumarins was reduced to below 0.2% upon their addition. In lemon essential oil, the presence of furocoumarins reduced the formation of the predominant Lim-2-OOH from 0.5% to 0.1%. Formation of *R*-(+)- imonene peroxides in lime oil started after a lag time of approx. 3 days for both, furocoumarin-depleted and furocoumarin-containing samples. However, the kinetics as deduced from the slope of the graph differed remarkably between the two samples. For lemon essential oil, the lag phase was significantly extended from about 4 to 7 days, and hydroperoxide formation was delayed (Fig. 4).

A low peroxide value of essential oils is crucial for the use of *R*-(+)-limonene as flavoring agent in cosmetic and personal care products. With a legal limit of only 10 ppm in leave-on products and 100 ppm in rinse-off products, *R*-(+)-limonene must be declared on the final consumer products as it is listed in the European Union Cosmetics Directive as potential fragrance allergen due to hydroperoxide formation [32]. Consequently, the shelf-life of

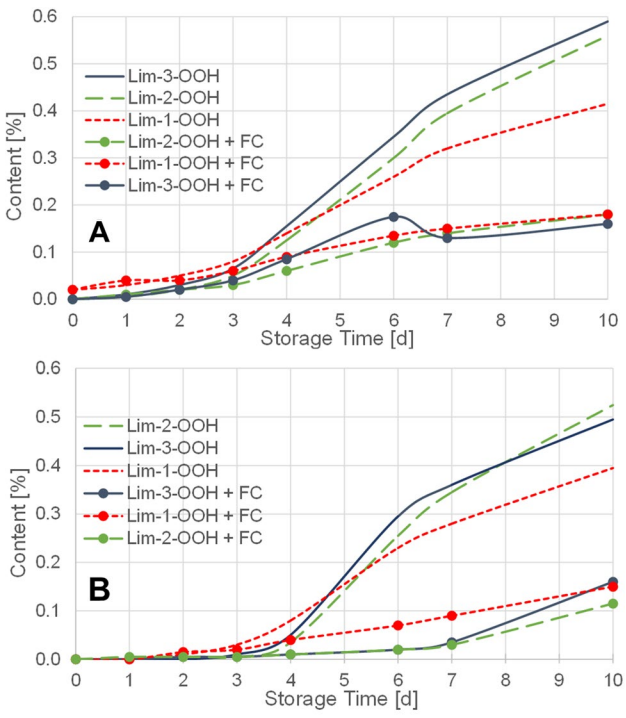

Fig. 4 Formation of limonene-hydroperoxides in lime (**A**) and lemon (**B**) essential oils during 10 days of storage under UV-A light. Samples containing furocoumarins are marked with dots on the graph

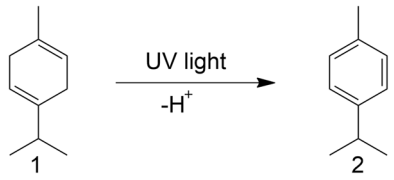

Fig. 5 Oxidative conversion (aromatisation) of γ-terpinene (**1**) into *p*-cymene (**2**) under UV light irradiation and proton splitting

essential agrumen oils containing furocoumarins may also be extended, as the *R*-(+)-limonene remains more stable within the oil matrix.

Effects of UV irradiation of essential oils on γ-terpinene and *p*-cymene contents

γ-Terpinene was also comparatively abundant in the three oils. This monocyclic monoterpene is extremely susceptible to dehydrogenation, thus yielding the aromatic follow-up product *p*-cymene (Fig. 5) [33]. Formation of *p*-cymene from γ-terpinene has also been described as a result of inadequate storage [34]. This is of particular relevance, because presence of *p*-cymene is linked with a kerosene-like off-flavor [35]. UV irradiation caused a steady and linear decrease in γ-terpinene contents with time in all three essential oils within 10 days (red line, Fig. 6A–C). Namely, in bergamot essential oil, the γ-terpinene concentration decreased from initially 6.4% to 1.0% without furocoumarin addition compared to 2.7% upon addition of the furocoumarin fraction isolated from bergamot oil (Fig. 5A). Vice versa, formation of *p*-cymene was reduced from 5.8% without furocoumarin addition to only 3.9% in the presence of furocoumarins (Fig. 5A). Hence, γ-terpinene was almost exclusively transformed into *p*-cymene. This effect was even more pronounced in lemon essential oil (Fig. 5B). Starting from an initial pool of 8.6% γ-terpinene, the compound was virtually completely dehydrogenated after 6 days of storage, thus forming *p*-cymene. In the presence of furocoumarins, however, 5.0% (1.9%) of the initial γ-terpinene was still present in lemon essential oil after 6 (10) days of UV light irradiation. Consequently, formation of *p*-cymene was also reduced in the presence of furocoumarins (Fig. 5B). With a share of 14.9%, lime essential oil showed the highest concentration of γ-terpinene at the beginning of the storage studies (Fig. 6C). In the absence of furocoumarins, this high share of γ-terpinene was almost completely transformed into *p*-cymene within 6 days. Noteworthy, Nguyen and coworkers showed that (rapid) loss of γ-terpinene was accompanied with massive off-flavor and loss of the fruity lemon freshness of the oils, especially when stored in the presence of oxygen [35]. For this reason, the protection of essential oil components susceptible to oxidation is essential, especially

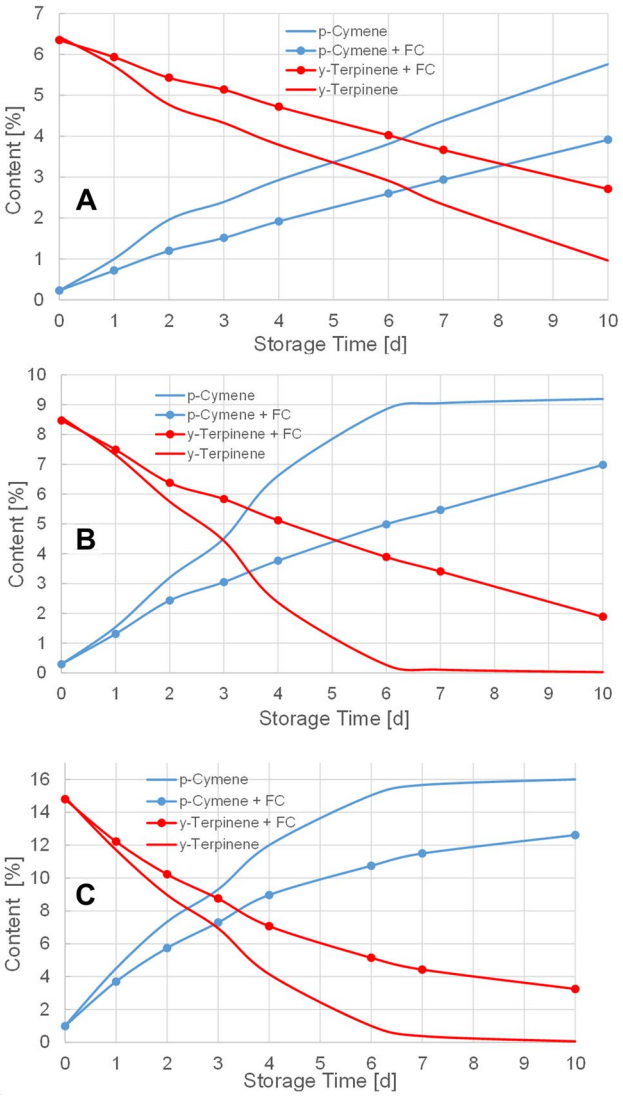

Fig. 6 Transformation of γ-terpinene into *p*-cymene in bergamot (**A**), lemon (**B**), and lime (**C**) essential oils during 10 days of storage under UV-A light. Both in the presence (dotted lines) and absence of furocoumarins

when they are used as flavoring agents in beverages, cosmetics and personal care products. Since addition of furocoumarins massively delayed the loss of γ-terpinene (Fig. 6C), it also lowered the formation of the off-flavor compound *p*-cymene (Fig. 6C), which had a beneficial impact on the sensory characteristics of the oil. After UV-A irradiation, samples with and without furocoumarin addition could be clearly distinguished olfactorily. Thus, samples devoid of furocoumarins showed more pronounced alterations of the compound profile, which is probably due to formation of other follow-up products. In addition to the conversion of γ-terpinene into *p*-cymene, secondary products of limonene such as carveols and epoxides may also influence the formation of undesirable off-flavors [35, 36].

Photo-protective actions of furocoumarins

As mentioned before, furocoumarins preferably absorb light in the UV-A range between 320 and 380 nm [37]. Very high molar attenuation coefficients of $\varepsilon_{250} = 3.03 \times 10^4$ and $\varepsilon_{250} = 2.42 \times 10^4$ L × mol^{-1} × cm^{-1}, respectively, were determined by Scheel et al. [23] for 8-methoxypsoralen and psoralen at 250 nm. In this study, we exemplified the molar attenuation coefficients for bergapten and 8-methoxypsoralen at 366 nm as this was the wavelength used in our UV irradiation experiments. The high values of $\varepsilon_{366} = 5.4 \times 10^3$ (bergapten) and $\varepsilon_{366} = 7.2 \times 10^3$ L × mol^{-1} × cm^{-1} (8-methoxypsoralen) confirmed the characteristics of furocoumarins to effectively absorb incident light which in turn most likely prevents photo-oxidation of other compounds. Such a protective effect of furocoumarins has been exemplified in this investigation for *R*-(+)-limonene and *γ*-terpinene. Accordingly, UV-A-sensitive terpenes are not directly affected by UV radiation, because furocoumarins act as protective shield, thus absorbing major parts of the radiation energy. Moreover, deactivation via fluorescence is linked with a strong bathochromic shift to wavelengths > 400 nm. Apparently, the resulting low-energy radiation is too weak to directly initiate terpene oxidation. In addition, furocoumarins can reach the triplet state by intersystem crossing (ISC). Hereby, energy emission to reach the ground (singlet) state via phosphorescence goes along with an even higher bathochromic shift up to 500 nm [38]. For instance, a light-yellow fluorescence (460 nm) and a yellow–green (490 nm) phosphorescence has been described for bergapten [39]. In both cases, high-energy UV radiation is transferred into visible light of lower energy, thus, strongly supporting terpene stability.

Conclusion

The photo-protective effect of furocoumarins on terpene stability could be verified by UV-A irradiation experiments performed with three citrus oils, i.e., bergamot, lemon, and lime. Specifically, the degradation of marker compounds such as *R*-(+)-limonene and *γ*-terpinene was effectively delayed. Consequently, the formation of undesirable oxidation products such as limonene hydroperoxides and off-flavor compounds such as *p*-cymene was clearly reduced. This is of considerable importance, since odor plays a decisive role in the application of essential oils in the perfume and cosmetics industry as well as in food industry.

Hence, natural furocoumarin levels in citrus and further essential oils not only increase their shelf-life but also improve their stability in final consumer products. Consequently, despite the phototoxicity of furanocoumarins, their overall rating should be re-evaluated with specific attention to structure–activity relationship as reported by Scott et al. [17]. Natural furocoumarin amounts as found in cold-pressed agrumen oils may therefore be of great benefit because of photo-protective effects on the essential oil, and fractions enriched in furocoumarins may be used as photo-protective agents added to highly sensitive essential oils.

Declarations

Conflict of interest The work was supported by WALA Heilmittel GmbH, Dorfstraße 1, D-73087, Bad Boll/Eckwälden.

Compliance with ethics requirements The study does not contain any studies with human participants or animals performed by any one of the authors.

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5 GENERAL DISCUSSION

5.1 Novel analytical approaches for terpene hydroperoxide detection

Although GC is still the method of choice in essential oil analysis it should not be forgotten that its application is linked with a number of analytical drawbacks and uncertainties [173]. For instance, the determination of low volatile, or compounds with molecular masses of about 300 Dalton are challenging and difficult to combine with the analysis of terpenes. Likewise, thermo-labile reaction products in essential oils may break down in the hot GC system, thus forming artifacts, and consequently escape detection [208,209]. In this context, terpene hydroperoxides play a crucial role because they are suspected to have a skin-sensitizing potential. Hence, their detection is highly relevant for the quality control of essential oils [2,210]. Terpene hydroperoxide analysis by GC can be accomplished after their reduction to the corresponding alcohols [127,189]. In addition, (trimethyl)silylation has been suggested as an alternative method for hydroperoxide analysis by GC [211,212]. As described by Rudbäck *et al*. (2014) the resulting trimethylsilyl (TMS) derivatives of all tested terpene hydroperoxides could be detected. Since the corresponding limit of detection (LOD) of 3,300 ppm (0.33%) is high, it is not sufficient for most applications [211].

This limitation of the GC method can be overcome by applying HPLC-DAD as well as HPLC-MS/MS approaches (section 3.7.2) [190,194,213]. However, UV detection in HPLC/DAD analyses has to be based on very low wavelengths (λ_{max} often below 200 nm) which makes it difficult to differentiate between individual terpenes [190]. By contrast, LODs of <1 ppm could be achieved by HPLC with electrospray ionization (ESI) using tandem mass spectrometric detection (MS/MS). The sensitivity of HPLC-ESI-MS/MS methods is sufficient to meet the low limits of 10 ppm for leave-on products stipulated in the EC regulation 1223/2009 on cosmetic products for potentially irritating and allergenic fragrance compounds [194,214]. However, investment costs for GC and HPLC equipment are high to make it applicable to any quality control laboratory. Furthermore, sample preparation is of utmost importance for the subsequent chromatographic analysis and is time-consuming and laborious [211,215]. Compared to that, iodometric titration of peroxides and hydroperoxides in essential oils is the current official method described in the European Pharmacopoeia [202]. Yet, in the presence of olefinic double bonds of accompanying compounds, this routine method

may lead to considerable problems in the analysis of hydroperoxides, since iodine can also be added to the former, which will result in incorrect values [216]. It is also known that the sample weight strongly impacts the result. Both problems negatively affect the overall accuracy of the iodometric titration method [216]. Alternative methods for the detection of other organic peroxides are still rare. In 1964, Banerjee and Budke applied a spectrophotometric method for the quantitative determination of iodine released from potassium iodide after a reaction with peroxides [217]. Also, decades ago Levy and co-workers presented a spectrometric method for lauroyl and benzoyl peroxides by derivatization with *N*,*N*-dimethyl-*p*-phenylene diamine sulfate. However, the LOD was both poor (~10 µg/mL) and strongly dependent on the pH value of the solution [218].

Consequently, a hitherto new approach making use of spectrophotometry for the detection of terpene hydroperoxides in essential oils was established in the present work. The goal was to minimize required sample amounts and to speed up and simplify sample preparation. The method should be applicable in routine analysis and be more economical than the iodometric titration, especially regarding high-priced essential oils. Also, the use of expensive and extensive GC or HPLC equipment should be avoided. In previous studies, various *p*-phenylenediamine derivatives including their sulfates and chlorides had been used for the detection of organic and inorganic peroxides [216,217,219]. The instability of the reagent, the slow reaction rate, and the lacking sensitivity are critical aspects in this context [217]. The investigations started with the synthesis of *cis*- / *trans*-limonene-2-hydroperoxide, which were used as terpene hydroperoxide standards in subsequent experiments (**paper 1**). The relatively stable limonene-2-hydroperoxides have often been detected upon limonene oxidation and in essential oils containing the latter [127,157,162]. Therefore, it was considered a suitable representative for subsequent investigations. Since, for both limonene-2-hydroperoxide isomers, NMR data of Karlberg *et al.* (1994) and Kao *et al*. (2011) were incomplete [153,220], additional details on signal multiplicities and coupling constants were determined by performing non-decoupled HSQC and 1D TOCSY experiments. Full NMR data are reported in Table 2 in **paper 1** and are illustrated in Figures 17 and 18.

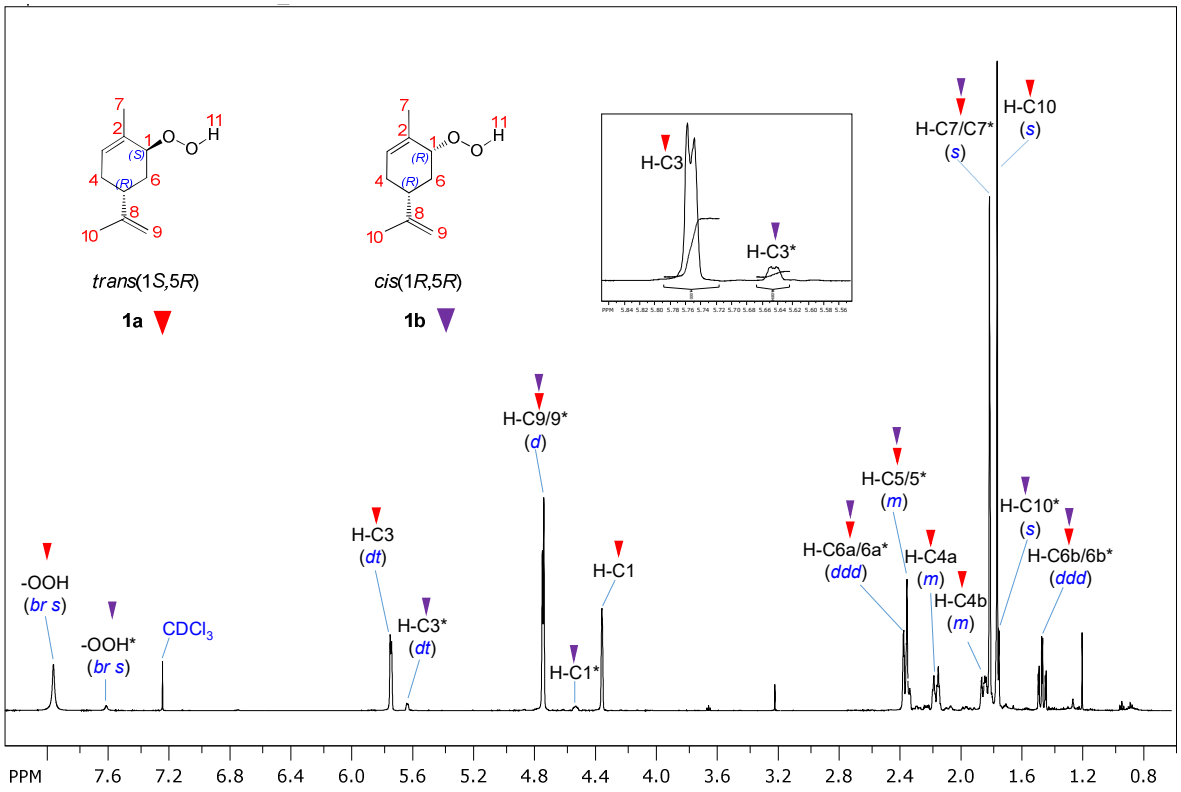

Figure 17. ^{1}H NMR spectrum of the synthesized limonene hydroperoxide (mixture of two stereoisomers **1a** and **1b**, **paper 1**), showing all proton signals. By integration of the signal areas of both H-C3 signals (see insert) the 1*S*/5*R* : 1*R*/5*R*-isomer ratio was found to be 92 : 8.

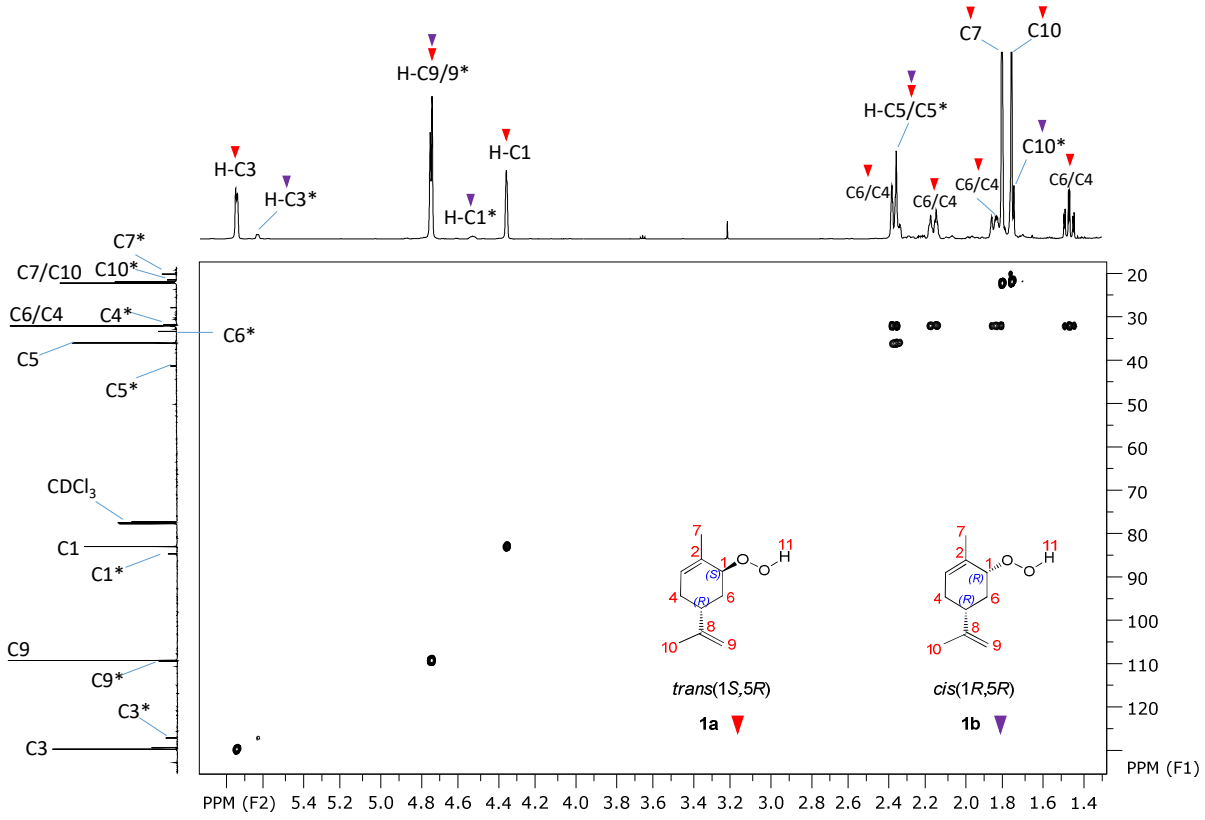

Figure 18. Gradient Heteronuclear Single Quantum Coherence (gHSQC) spectrum of the synthesized limonene hydroperoxide isomer mixture (**1a** and **1b**, **paper 1**). gHSQC is a two-dimensional inverse ^{1}H-^{13}C correlation technique that allows the determination of carbon to hydrogen connectivity for full structure elucidation.

The synthesized limonene hydroperoxides and two commercially available hydroperoxides (i.e. cumene hydroperoxide and dibenzoyl peroxide) revealed that *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) was the most suitable reagent. DMPD is oxidized by peroxides to yield resonance-stabilized cations which display a strong red color and are a.k.a. Wurster's red [221,222]. The intense color is predestined for spectrophotometric detection. Therefore, the method was validated according to the ICH Q2(R1) guidelines employing spectrophotometric determination at λ_{max} 517 nm ensuring it to be robust and reproducible (**paper 1**). Further peroxidized terpenes and essential oils were prepared by using rose bengal as a photo-catalyst. Samples were irradiated with an LED lamp at 6500 K and 500 lumen (lm) to ensure constant conditions. In the presence of photosensitizers, such as rose bengal, a fast photo-oxidation of double bonds by singlet oxygen has often been described in the literature [127,153,162]. The result of the spectrophotometric analysis of the oxidized terpenes was in good correlation with literature findings reporting their presence in distinct essential oils. Relatively stable terpene hydroperoxides were reported for β-citronellol [223], linalyl acetate [211], limonene [127,153], linalool [210,224], citral [139] and valencene [225]. However, hardly any hydroperoxides were detected in the case of carvone despite the presence of two C=C double bonds (Figure 19, **6**). This terpenoid is known to be formed upon oxidation of limonene via the decomposition of limonene hydroperoxides (Figure 19) [2,153,226]. The photochemical cleavage of carvone may also be stabilized by the Norrish-Type-I reaction scheme. The cleavage of the α-carbon bond of ketones may be re-arranged by a racemization reaction to the initial molecule (see **paper 1**, Figure 5) [227,228]. The photo-experiments presented in **paper 1** confirmed these results.

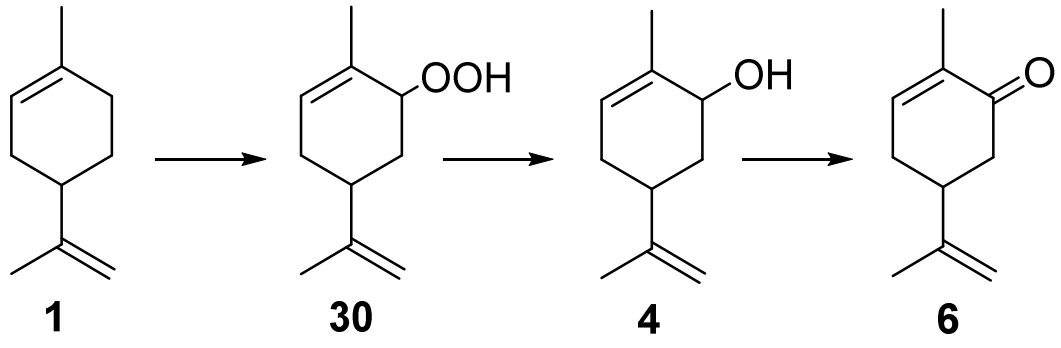

Figure 19. Formation of carvone (**6**) via limonene hydroperoxide (**30**) and carveol (**4**) from limonene (**1**). The oxidation of limonene is described by a free radical mechanism that is not explicitly shown here [155].

It was shown in various test systems that the spectrophotometric method could also be applied to essential oils, representing multicomponent mixtures (**paper 1**). Initially, the freshly obtained oils did not show any hydroperoxide formation. Yet, irradiation of lemon oil and caraway oil, both having high contents of limonene, was accompanied by the formation of a notable amount of peroxides. These results confirmed the sensitivity of the method and qualified it for routine analysis in the field of essential oils and essential oil-containing consumer products (**paper 1**). Furthermore, photo-oxidation of limonene strongly differed from thermally induced alteration. In the latter process, hydroperoxides played only a subordinate role. Instead, various other compounds including alcohols and epoxides were detected that have also been described by McGraw *et al*. (1999) and Kiralan *et al*. (2021) [229,230]. Nevertheless, both processes usually take place concomitantly during the storage of samples [150]. The thorough examination of the structurally diverse oxidation products led to the idea of performing gravimetric experiments. Assuming complete hydroperoxide formation of a terpene with a molar mass of 136 g/mol ($C_{10}H_{16}$), the molecular weight will increase by 32 g/mol. In case of an initial amount of 1.60 g of this terpene used for photo-oxidation experiments as described in **paper 1**, this corresponds to a mass of 1.98 g if this terpene is completely converted into a hydroperoxide, which is equivalent to a gravimetric increase by 0.38 g or 23.8%, respectively. Therefore, the mass increase may be easily detected by means of a fine balance.

In the investigations presented here, the weight gain was sometimes considerably high and suggested strong hydroperoxide formation. For instance, the weight gain of citral was almost 40%. Also, the introduction of a so-called oxidation factor enabled the comparison of different samples with each other (**paper 1**). Currently, the oxidation factor can only be considered in the case of complete conversion into hydroperoxides. Otherwise, the expression strength is only a guideline, since ketones and alcohols have a lower molecular weight than other terpenoids. Nevertheless, the results of the gravimetric method were in good correlation with spectrophotometric experiments and GC data. This gravimetric procedure described in **paper 1** may also be a helpful tool in routine essential oil analysis to get a first impression of the state of oxidation or alteration during storage, with valuable information on a long-term basis if results are fed in a database. Perceivable weight changes during essential oil storage could thus serve as a low-effort starting point for further investigations. Hereby, additional density measurements and their utilization together with gravimetric data could be of particular

interest. Altogether, the spectrophotometric method introduced in **paper 1** represents a fast and accurate measurement system for the detection of hydroperoxides in terpenes and essential oils. Application opportunities are manifold and may be supportive compared to titration and time-consuming and expensive GC and HPLC analyses.

5.2 Matrix effects on the aging behavior of terpenes and essential oils

Quality monitoring of essential oils has become increasingly important since their production has been industrialized for covering increasing demands in different fields (section 3.5). The steadily increasing consumers´ interest in natural ingredients has been accompanied by many scientific studies on the stability of terpenes [161,229,231], essential oils [150,152,158,232], and plant materials containing them [233,234]. These investigations covered the impact of parameters such as heat, oxygen, radiation, and catalytic agents on the autoxidation and photo-oxidation of terpenoids during storage [2]. However, all previous storage studies were based on different samples of the same type. Namely, terpenes were compared with terpenes [229] or essential oils with other essential oils [152]. Detailed knowledge on storage and aging effects of terpenes forming part of the extracted essential oil compared to the entire plant matrix on the one hand or the neat substances, on the other hand, had been lacking.

For this reason, a detailed storage study was conducted, which is presented in **paper 2**. Specifically, the pure terpenes limonene and carvone, caraway essential oil, as well as caraway seeds, were examined in a storage experiment over the course of 18 months. The caraway essential oil was obtained directly after its production from freshly harvested seeds on a local farm. Thus, the entire supply chain was available (fresh plant material, seeds, and essential oil) for this investigation (**paper 2**). In addition, a commercial caraway essential oil stored for one year was included in this study. Caraway was selected because of the small size of its seeds. These allow a representative sample withdrawal even in the case of small sample amounts. Moreover, the essential oil can be easily obtained in adequate amounts from the seeds by laboratory-scale hydro-distillation (**paper 2**). In addition, caraway is a widely used essential oil in the pharmaceutical, cosmetics, and food industries [112]. Furthermore, 90% of the entire essential caraway oil consists only of *R*-(+)-limonene and *S*-(+)-carvone. Both terpenes were also available as single compounds in adequate amounts and purity as well as high enantiomeric purity [97,235]. The seeds were stored in small cotton bags which were sewn for this purpose (**paper 2**, Figure 2). This realistic setup also allowed the gas exchange with the caraway seeds similar to conditions during commercial storage of this product [236]. Liquid oils and terpenes were stored in half-filled 2 mL white glass vials that resembled the normal scenario with air space above the samples (**paper 2**). The relative humidity (approx. 43%, facilitated with

supersaturated potassium carbonate [237]) and temperature (25 °C and 40 °C) agreed well with conditions reported by Rajamanickam *et al*. (2013), correlating with an equilibrium moisture content for caraway seeds of ~5% at 25 °C and 9% at 40 °C, respectively [238]. Under these conditions, the formation of mold could be circumvented, which may have influenced compound composition due to enzymatic reactions [239]. The water content of 8% in the caraway seeds (determined according to the European Pharmacopoeia) was within the range of <15%, which is required for their transportation [236]. Stored samples were analyzed by both GC/MS and HPLC-DAD-APCI-MS/MS which allowed us to detect virtually all changes occurring during storage (**paper 2**). Despite the predominance of *R*-(+)-limonene and *S*-(+)-carvone (>90%), GC/MS measurements enabled the detection of 26 compounds in the caraway essential oils. This variety is between the maximum number of 49 compounds determined by Solberg *et al*. (2016) and only 16 terpenes reported by Salveson and Svendsen (1976) in caraway essential oil [240,241].

Quantitation was carried out with GC/FID, which is a robust detection method characterized by a good sensitivity towards organic compounds [1,174]. This resulted in very small standard deviations (n=3) for all compounds in the different samples (**paper 2**, Table 1). Newly formed secondary products such as alcohols, ketones, and epoxides were unequivocally identified by GC/MS (**paper 2**). In contrast, the identification of terpenes by HPLC-MS was less successful, since the mass spectra were more difficult to interpret and fewer reference data was available. However, more polar oxidation products such as limonene-1,2-epoxide and carvone-1,2-epoxide were successfully characterized by HPLC-APCI-MS/MS. Consequently, all changes in terpene standards, seeds, and essential oil samples could be tracked by the combined application of HPLC and GC (**paper 2**). The terpene alteration in the storage study visualized by this approach most probably proceeded via thermally induced autoxidation that describes a radical chain reaction pathway (section 3.6.1) [2]. Hereby, many compounds were formed that differed from the array of compounds observed after photo-oxidation, which are reported in **paper 1**. The rose bengal catalyzed oxidation of terpenes and essential oils predominantly resulted in terpene hydroperoxides (**paper 1**). In agreement with Schieberle *et al*. (1987), the oxidation of limonene led to six hydroperoxides (**paper 1**) [127], which were also observed during the photo-oxidation of lemon essential oil (**paper 1**) [157]. Accordingly, the long-term storage experiment revealed the formation of primary autoxidation products along with

several further secondary oxidation products for limonene and caraway essential oil (limonene content 47%). Characteristic abundant compounds were, among others, *cis*- and *trans*-limonene-1,2-epoxide, *cis*- and *trans*-carveol, as well as carvone. In the longer term, limonene-1,2-diol, formed by the degradation (ring opening and addition of water) of limonene epoxide, reached highest amounts as an oxidation product in the neat terpene standard after 18 months (**paper 2**). This could be quantitated with the help of a reference standard generated by alkaline treatment of (previously synthesized) limonene-1,2-epoxide (**paper 2**). These versatile oxidation products resulting from limonene are also illustrated in detail in Figure 12 in section 3.6.

The results of **paper 1** and **paper 2** clearly show that storage-induced aging can be traced back by means of different secondary oxidation products. The absolute amount and ratio of secondary marker substances limonene-1,2-epoxide to limonene-1,2-diol led to a robust evaluation of the progress of aging (**paper 2**). Remarkably, the degradation of terpenes in the stored essential oil was slower as compared to the neat substance. Protection and stabilization of individual terpenes may be attributed to other compounds in the essential oil. Contrary to the progression in the essential oil, degradation of pure limonene proceeded nearly linearly over the entire storage period. No interactions with other compounds, having a mutual stabilizing effect, were possible, and apparently, the formed hydroperoxides did not play a role in this context. The protective effect of the essential oil matrix was even more pronounced in caraway seeds. No changes could be noticed after 18 months of storage at 25 °C and 40 °C, since oxidation products were not detected at all. Within the plant matrix loss of terpenes due to evaporation was also low throughout the entire storage period (**paper 2**). The observed high stability agreed with the previous results of Fehr *et al*. (1980) who noted a loss of merely 10% of essential oil from caraway seeds after 44 months of storage [242]. Compared to that, loss of essential oil from fennel seeds amounted to 38% in the same period [242].

Besides the matrix, the compounds themselves play a key role in the stability of essential oils upon storage (**paper 2**). For instance, the terpenone carvone was highly stable under both photo-oxidative conditions (**paper 1**) even at elevated temperatures over a storage period of 18 months (**paper 2**). However, this could be due to the fact that carvone (and other oxidation products) was formed from limonene by oxidation during storage at 40 °C (**paper 2**, Figure 6). Furthermore, with regard to the follow-up

products, the alteration may be regarded as a stepwise progression. The final oxidation products were also the compounds with the highest amount of oxygen, as for instance carvone-epoxide or limonene-1,2-diol (**paper 2**) [2,139]. The formation of hydroperoxides may also occur during the storage of limonene and caraway essential oil, but may be directly decomposed due to the long storage time at elevated temperatures. This finding was described in detail by Turek and Stintzing using various essential oils [152,160]. Namely, the peroxide value determined by titration (section 3.7.4) increased in the beginning but flattened towards the end of the storage period (12 weeks, 38 °C). In contrast, the pH value decreased and the conductivity increased over time most likely due to the conversion of hydroperoxides into secondary oxidation products [152,160]. The degradation of limonene and the formation of secondary oxidation products could also be perceived as an off-flavor. This is of immense importance since odor is the key reason for the use of essential oils in most products such as cosmetics and household cleaners. This also demonstrates that human sensory perception should not be underestimated even though various high-tech analytical devices are available in research today.

In the field of flavor and essential oil analysis, sensory evaluation still plays an important role e.g. in finished product evaluation. This can be supported on a molecular level by using gas chromatography with olfactometric detection (GC/O). In GC/O systems the end of the capillary column is connected with a sniffing port, where humans can perceive the odors of the eluting compounds individually [180,181,243]. In this way, not only odor-active compounds can be perceived, but also their contribution to the overall smell can be determined in combination with dilution experiments (a.k.a. flavor dilution analysis) [180,181]. This in turn may lead to the identification of character impact compounds [243]. Typically, these character impact compounds have a major influence on the overall impression of essential oils, like eugenol in the case of clove essential oil, and should therefore receive special attention during the stability evaluation of essential oil-containing samples [61].

5.3 Interactions of volatile and non-volatile essential oil compounds

As described before, the oxidation of fragrance compounds is a key problem of essential oil and terpene storage (**paper 1**). Due to the formation of unwanted follow-up products associated with it, such processes need to be limited, also because some oxidation products, such as distinct terpene hydroperoxides, are potentially allergenic and are known to cause off-flavor [139,153,244]. At the same time, different studies have reported the use of essential oil-containing plant extracts as natural antioxidants in recent years [245,246]. For instance, rosemary extract has been applied both in the food and cosmetics industries as a replacement for synthetic ingredients such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) [247,248]. Typically, the antioxidant and preservative mode of action is owed to the quenching of peroxyl radicals, thus decelerating autoxidation [108,111]. On a molecular level, positive effects could be traced back to the presence of carnosic acid and/or phenolic compounds such as rosmarinic acid and flavonoids as well as eucalyptol (1,8-cineole) [248–251]. Similarly, terpenoids such as carvacrol and thymol, which are present in distinct amounts in thyme and oregano essential oils, were also shown to be potent antioxidants [108]. Furthermore, Ye *et al*. (2013) found that the addition of eugenol exerted protective effects on the thermal stability of cinnamon (*Cinnamomum osmophloeum* Kaneh.) essential oils [159]. In this case, one specific compound was able to protect essential oil compounds from degradation [159]. Accordingly, plant-specific natural components may be applied to foods and natural cosmetics, thus underlining the aspect of the naturalness of the products, with the additional benefit of a longer shelf-life.

Most essential oils together with their antioxidant components are gained from the raw material by making use of their high volatility (section 3.3). In this context, agrumen oils are different because they are usually obtained by cold-pressing (section 3.2.3). Hence, the resulting extracts also feature non-volatile compounds including furocoumarins (section 3.3.4). Prior to the experiments presented in **paper 4**, the effect of furocoumarins on the protection of essential oils had not been explored. Typically, furocoumarins are considered rather unwanted by-products of essential oils [18,41]. Dugo *et al*. (1997) reported concentrations of more than 3 g/100 g of the furocoumarins bergamottin and 5-(geranyloxy)-7-methoxycoumarin in cold-pressed lime essential oils

[41]. Similarly, high furocoumarin amounts (4.9 weight-% in lime, 1.3 weight-% in bergamot essential oil) were determined in the study presented in **paper 4**.

Undesired effects of furocoumarins include phototoxicity, which may cause sunburn-like skin reactions under the impact of sunlight [71,75,252]. Also, carcinogenicity has been reported for individual furocoumarins due to potential interaction with DNA or inhibition of CYP P450 enzymes [70,75,253]. Furocoumarins are photo-reactive because they can absorb UV light between 320 and 400 nm (UV-A light) [72,75]. The bulk of the absorbed energy of excited molecules can be released via fluorescence at wavelengths between 380 to 600 nm. Moreover, excited furocoumarins can be converted into the triplet state by intersystem crossing (ISC) followed by the emission of phosphorescence at even higher wavelengths from 450 to 600 nm (section 3.3.4, Figure 9). Accordingly, high-energy UV light is transferred into visible light of lower radiation energy [71]. This physicochemical property led us to the basic assumption that furocoumarins may pose a photo-protective and shielding effect on surrounding components by transforming high-energetic UV light into less reactive visible light. The systematic investigation of potential effects was initially studied with commercially available furocoumarin and terpene standards regularly found in many well-known essential oils, i.e. β-pinene, *R*-(+)-limonene, and γ-terpinene. In addition, these terpenes are also known to rapidly undergo oxidative changes. Specifically, β-pinene and *R*-(+)-limonene are known to quickly form hydroperoxides, while γ-terpinene is easily converted into *p*-cymene via H^+ abstraction and aromatization (**papers 1** and **4**) [128,231,254].

For furocoumarins, only xanthotoxin (8-methoxypsoralen) and bergapten (5-methoxypsoralen) were commercially available in adequate amounts. Accordingly, the relevant furocoumarins bergaptol and bergamottin had to be synthesized first. Bergaptol was obtained from bergapten by demethylation with boron tribromide (BBr_3) [255]. An overall yield of 84% and an amazingly high purity of ≥ 99% were achieved after re-crystallization from *n*-hexane (**paper 3**). The step was implemented because it was assumed that bergaptol was more polar than bergapten and less soluble in *n*-hexane (**paper 3**). In a further step, an aliquot of the synthesized bergaptol was converted into bergamottin by reaction with geranyl bromide. This reaction also worked excellently under alkaline conditions using potassium carbonate in acetone. However, purification of the latter was challenging. Finally, impurities still present after column

chromatography could be removed in an additional purification step via centrifugal thin-layer chromatography using a Chromatotron system (**paper 3**). This system takes advantage of a round disc coated with a mixture of silica gel and gypsum as the stationary phase. It follows the application of the solvent during the rotary movement of the disc. It has previously been successfully used for the isolation of natural compounds such as triterpenoids or glycoalkaloids with high purities and quantities [256,257]. In the present case, the final purity of ≥ 90% was sufficient for NMR and HPLC-APCI-HRMS analyses (**paper 3**). NMR data of bergamottin were in good accordance with literature reports except for some differing signal multiplicities, which had been reported by Girennavar *et al*. (2005) [253]. Subsequent photo-oxidation experiments were performed with 5 weight-% furocoumarins added to neat terpenes (**papers 3** and **4**). This share was similar to the reported natural furocoumarin contents in citrus oils (**paper 4**) [18,41]. Irradiation experiments were performed with solutions in ethanol (100%). Ethanol was chosen because of the high solubility of furocoumarins in this solvent. Furthermore, a strong photo-reaction of the furocoumarins had been observed using this solvent [258]. The use of a Desaga photo chamber allowed a homogenous illumination without undesired disturbing and fluctuating daylight.

In agreement with previous studies, irradiation of terpenes without furocoumarins resulted in a very strong degradation and formation of several secondary products (**papers 1** and **3**) [127,128,244]. It was striking that γ-terpinene was exclusively converted into the more stable, aromatic *p*-cymene (**paper 3**) [254]. On the contrary, irradiation of limonene led to a distinct formation of hydroperoxides (**papers 1** and **3**). Similarly, β-pinene was converted into hydroperoxides, along with various alcohols (**paper 3**). When the experiments were repeated in the presence of furocoumarins, a strong protective effect was observed in all cases (**paper 3**). The overall degradation of the three terpenes was substantially reduced and the furocoumarin structure influenced the reaction products (Figure 20). Among all investigated terpenes, the strongest photo-protective effects in the presence of UV-A light were observed for bergaptol and xanthotoxin (**paper 3**). Furthermore, the stability of the furocoumarins also played a crucial role. Lower degradation rates of furocoumarins naturally go along with enhanced protection of terpenes from UV light. Specifically, after ten days of UV irradiation only 20% of the initial amount of bergaptol was lost, while bergapten was strongly degraded (loss of 90% in the same period) (**paper 3**). Subsequent UV-A

irradiation for 20 days with pure furocoumarin solutions in ethanol and without terpenes confirmed the instability of both bergapten and bergamottin (**paper 3**). The UV light stability of furocoumarins also plays an important role especially when they are used as a therapeutic agents for the medical treatment of vitiligo or psoriasis [259]. In this field of application, the furocoumarins are expected to retain their efficacy over the whole time they are applied to the skin and irradiated with UV-A light [260].

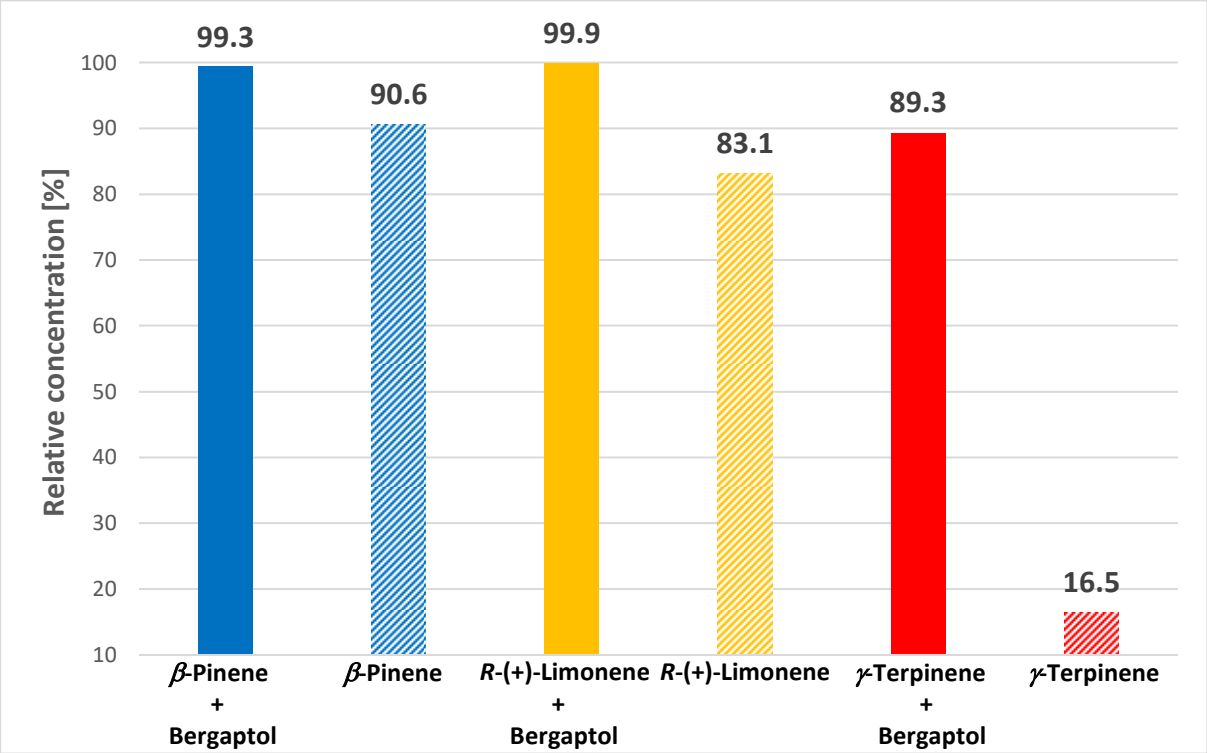

Figure 20. The relative concentration of β-Pinene, *R*-(+)-Limonene, and γ-Terpinene containing 5% Bergaptol (full bars) in comparison to the amount without Bergaptol addition after ten days of UV-A light irradiation at 366 nm. The numbers above the bars indicate the relative retention of terpenes (%) at the end of the experiment.

After these promising results with furocoumarins and terpenes, further experiments were performed to explore the potentially protective role of furocoumarins in essential agrumen oils. For this purpose, furocoumarins were initially isolated from three agrumen oils (lime, lemon, and bergamot). Specifically, 150 g essential oil was mixed with 800 mL *n*-hexane and cooled to -80 °C. Under these conditions furocoumarins started to crystallize and subsequently could be filtered off reaching purities of >97% according to GC/FID analysis (except bergamot essential oil, where the furocoumarin fraction additionally featured linalool and linalyl acetate). The resulting furocoumarin

profile of the isolates agreed well with that of the agrumen oils (**paper 3**). In the following, three samples dissolved in ethanol were irradiated, i.e. (i) the natural essential oil, (ii) the natural essential oil freed from furocoumarins as shown above, and (iii) aliquots of the natural essential oil freed from furocoumarins supplemented with 5 weight-% of the respective furocoumarin. Due to their high amounts in all essential oils, the fate of *R*-(+)-limonene and γ-terpinene was monitored during photo-oxidation. In lime and lemon essential oils freed from furocoumarins, *R*-(+)-limonene was massively degraded within ten days. Previous studies have shown that UV-A light-induced degradation of *R*-(+)-limonene in lemon oil was accompanied by a loss of the favorable fresh and fruity citric smell. *Vice versa*, turpentine, and solvent-like off-flavor notes became dominant [150,157]. In the absence of furocoumarins, limonene was converted into limonene hydroperoxides (**papers 3** and **4**). Under these conditions, (2*S*,4*R*)-*p*-mentha-6,8-diene-2-hydroperoxide (**31**), (2*R*,4*R*)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide (**32**), and (1*R*,4*R*)-*p*-mentha-2,8-diene-1-hydroperoxide (**33**) contents amounted to >0.5% in the essential oil (Figure 21). At such levels, hydroperoxides have been suspected to cause allergic reactions [213,231].

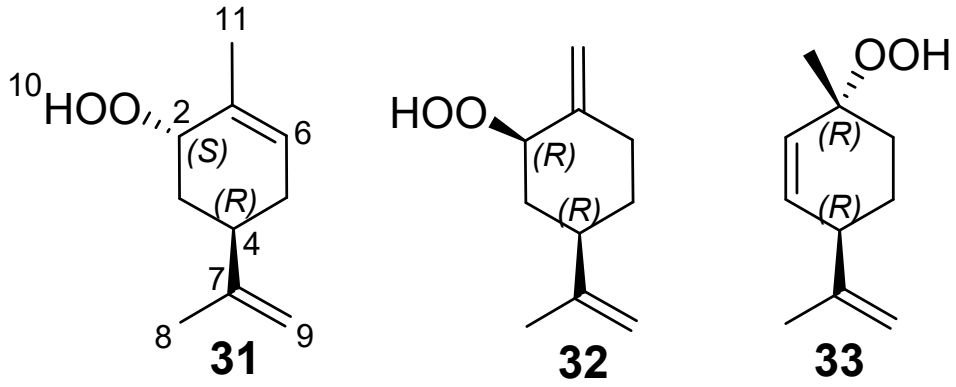

Figure 21. Main photo-oxidation products (2*S*,4*R*)-*p*-mentha-6,8-diene-2-hydroperoxide (**31**), (2*R*,4*R*)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide (**32**), and (1*R*,4*R*)-*p*-mentha-2,8-diene-1-hydroperoxide (**33**) found in lime and lemon essential oils devoid of furocoumarins after irradiation with UV-A light for ten days.

These observations were substantiated by γ-terpinene. In the absence of furocoumarins, it was quantitatively converted into *p*-cymene after seven days of UV light irradiation in lemon and lime essential oils. This was remarkable considering the initial contribution of 15% to lime essential oil. *Vice versa*, the amount of *p*-cymene was increased in the same range. In this context, it is worth mentioning that γ-terpinene has

often been reported to be easily oxidized and converted into *p*-cymene. This finding has been reported not only for essential oils but also for the neat substance [150,157,254]. The resulting kerosene- and turpentine-like off-flavor was mainly responsible for a quality reduction of the corresponding essential oil [150].

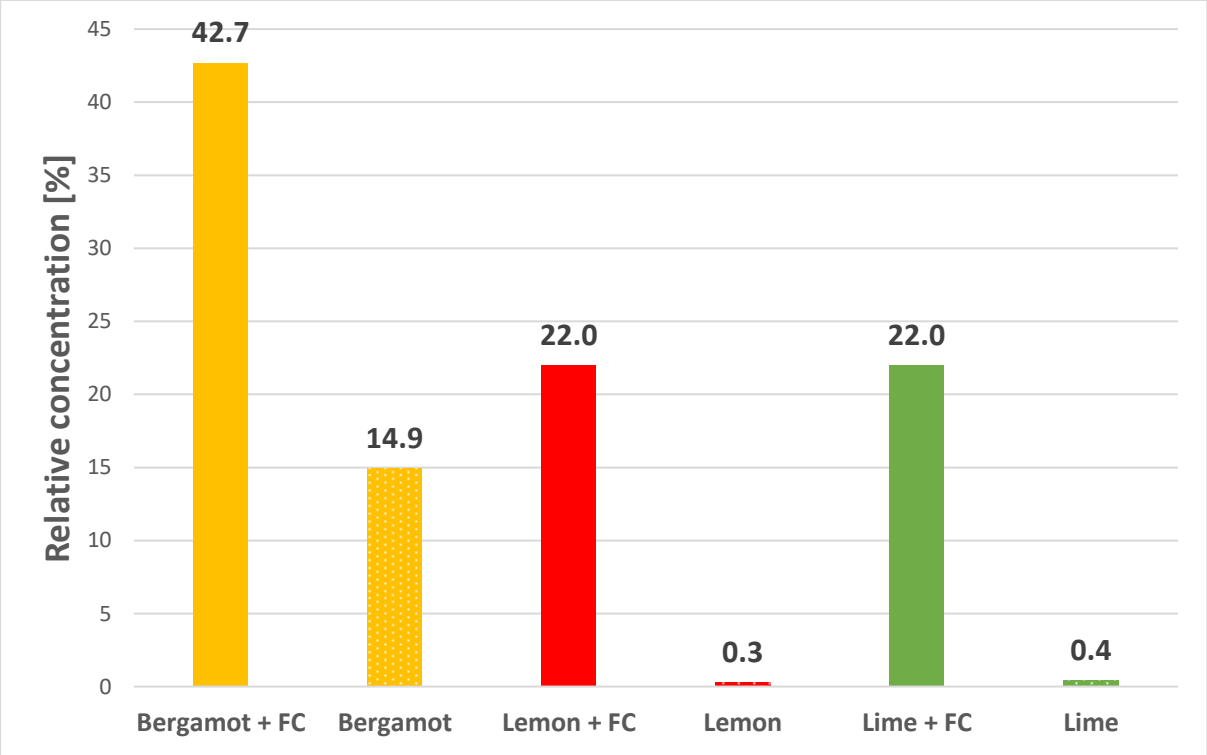

Figure 22. Remaining share of γ-terpinene in bergamot, lemon, and lime essential oils upon UV-A irradiation for ten days at 366 nm. The full bars show the natural furocoumarin containing essential oils (+ FC), whereas the dotted bars show the essential oils devoid of furocoumarins. The numbers above the bars indicate the relative retention of terpenes (%) at the end of the experiment.

The need to protect these sensitive oils from oxidation together with the call for “naturalness” in the food and cosmetics industries [261] prompted us to study if furocoumarins may provide such an effect in essential oils, similarly as observed with neat terpenes in **paper 3**. Remarkably, the addition of the separated furocoumarin fraction to the three essential agrumen oils initially freed from furocoumarins lowered the degradation of *R*-(+)-limonene to a maximum of 2.3% in lime essential oil and even less for lemon (0.7%) and bergamot (0.1%) (**paper 4**). A high level of protection was observed with all furocoumarins (**paper 4**). Also, the differences were small between the individual structures, which is in agreement with the data presented in **paper 3**. In the case of γ-terpinene (Figure 22), the addition of the furocoumarin fractions reduced

its degradation rate over the entire period and decelerated its conversion into *p*-cymene (**paper 4**).

Besides improved organoleptic properties, the addition of furocoumarins to the essential oils massively reduced both the degradation of *R*-(+)-limonene (Figure 23) and the formation of hydroperoxides. The overall maximum concentration of hydroperoxides was below 0.2% and, thus, almost three times lower than in the absence of furocoumarins.

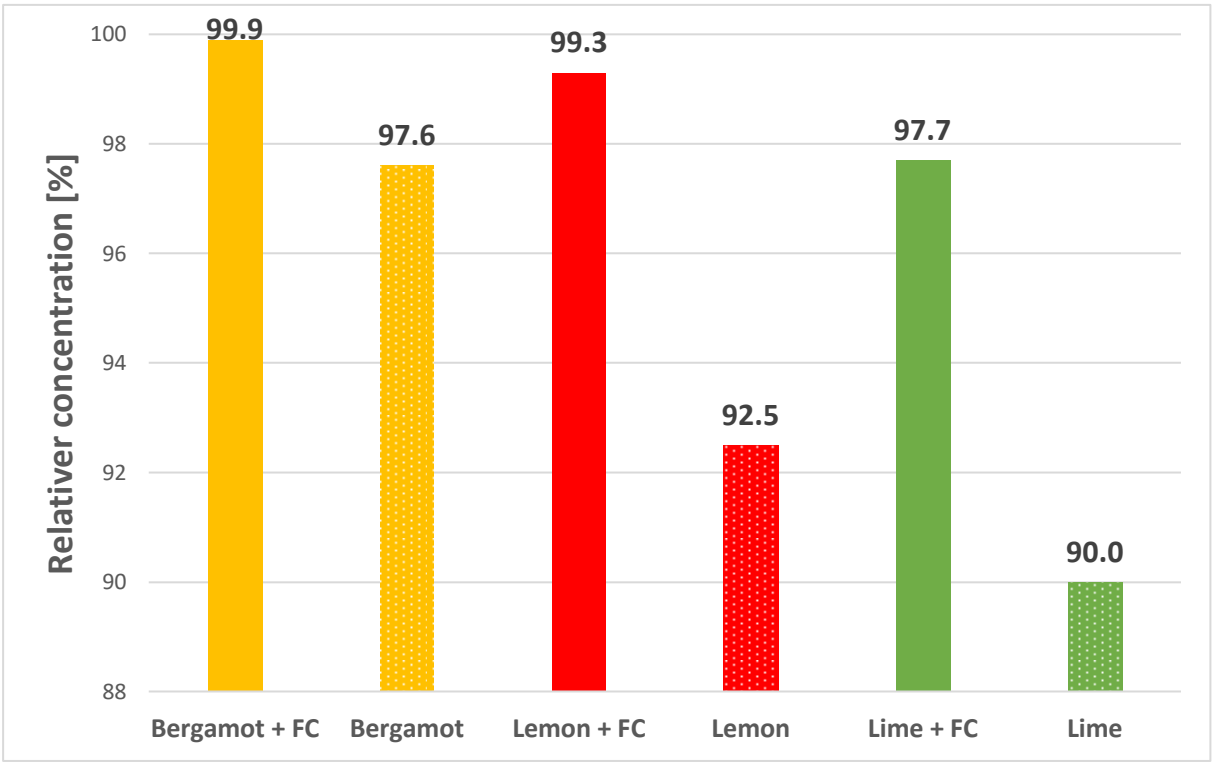

Figure 23. Remaining share of *R*-(+)-limonene in bergamot, lemon and lime essential oils upon UV-A irradiation for ten days at 366 nm. The full bars show the natural furocoumarin containing essential oils (+ FC), whereas the dotted bars show the essential oils devoid of furocoumarins. The numbers above the bars indicate the relative retention of terpenes (%) at the end of the experiment.

Accordingly, furocoumarins displayed a remarkable photo-protective effect on the three terpenes (Figures 22 and 23). Therefore, they may be used as natural preservatives to increase the shelf life of terpenes, essential oils, or final consumer products (**papers 3** and **4**). Yet, potential risks of photo-toxic skin reactions need to be studied in more detail. Based on these unequivocal findings, furocoumarins should not be demonized *a priori*. Rather, the aim is to initiate a critical debate on their occurrence in essential oils, thus taking a closer look at their structure-based reactions in terms of

phototoxicity as well as photo-protective effects as presented by Scott *et al*. (1976) [72,262].

5.4 The variability of volatile organic plant compounds

As described in **papers 1-4** essential oils are exposed to different extrinsic factors that may have both positive and negative effects on their quality. For instance, temperature, light, and the presence of oxygen directly affect individual essential oil components with the effect being dependent on their chemical structure [2]. As an example, citronellol and *R*-(+)-limonene were much more prone to oxidation than *S*-(+)-carvone or valencene (**paper 1**). Also, some unstable substances are converted into different primary and secondary reaction products. Typically, light-induced photo-oxidation predominantly resulted in the formation of hydroperoxides (**papers 1**, **3**, and **4**), whereas autoxidation at elevated temperatures led to a variety of different secondary oxidation products including alcohols, ketones, epoxides, and diepoxides (**paper 2**). However, a clear distinction between both reaction pathways is not always possible in essential oils stored over a longer time or when forming part of consumer finished products. Yet, detrimental and irreversible effects on product quality make it necessary to take action at an early stage. For this purpose, the influence of storage conditions, packing materials as well as the addition of antioxidants had been the subject of previous studies [150,160,263].

However, the possible impact of the plant matrix itself had been mostly overlooked before. In this context, the enormous protective effect has been exemplified by means of a storage study with caraway seeds over 18 months (**paper 2**). This protective effect of the matrix could be verified in **paper 2** by comparing (i) freshly distilled essential oil (from stored seeds), (ii) the essential oil stored over this long period, and (iii) similar experiments with neat compounds (*R*-(+)-limonene and *S*-(+)-carvone). In accordance with Fehr *et al*. (1980), the loss of essential oil components was effectively prevented as long as they formed part of untreated seeds [242]. Similarly, the stabilizing effects of furocoumarins on essential oil components was systematically investigated by means of their removal from and subsequent addition to the essential oil (**papers 3** and **4**). However, representative furocoumarins (bergapten, 8-methoxypsoralen, bergaptol, bergamottin) are only present in citrus fruits and their essential oils (agrumen oils), since these are not obtained by distillation but extraction [191,264,265]. These results showed that not the entire plant matrix was required to obtain protective effects, but that selected compounds or compound classes such as furocoumarins may be similarly effective (**papers 3** and **4**). These findings open the door for further

protective actions that may be investigated aiming at the protection of essential oils and their further quality improvement. These findings also clearly demonstrate that the stability and chemical behavior of isolated substances cannot be transferred to essential oils as multicomponent mixtures. Finally, the selective removal of compounds from such natural mixtures may come along with negative consequences as shown for the furocoumarin-depleted agrumen oils investigated in this thesis.

6 SUMMARY

Essential oils belong to secondary plant metabolites, with terpenoids and phenylpropanoids being among the main constituents in terms of quantity. Due to their lipophilic character and high volatility, they are mainly obtained by steam distillation. Citrus essential oils (agrumen oils) are an exception , since they are usually extracted from the peels by means of pressing, whereby less volatile components such as coumarins and furocoumarins are also introduced. Due to their odor and taste-giving properties, essential oils are used in the food, beverage, and cosmetics industries. In addition, due to a wide range of pharmacological properties, they are used in phytotherapy as well as in aromatherapy. However, most essential oils are highly susceptible to oxidation, polymerization, dehydrogenation, and isomerization reactions in the presence of atmospheric oxygen, light, and at high temperatures. The resulting organoleptic changes usually lead to a significant quality reduction. The formation of terpene hydroperoxides is another problem, as these are suspected of causing intolerances such as redness and itching in 1-3% of the European population upon contact with the skin. The detection of these chemical changes forms an integral part of quality control and can be prevented as far as possible by suitable production, transport, and storage strategies. Due to their volatility, essential oils are mainly analyzed by gas chromatography. However, due to their instability, the detection of hydroperoxides places considerable demands on common analytical methods. For this reason, a novel spectrophotometric method for the detection of peroxides and hydroperoxides in terpenes and essential oils was developed (**paper 1**). The oxidation of *N*-*N*-dimethyl-*p*-phenylenediamine by peroxides yielding an intensely red-colored cation (Wurster's red) allowed colorimetric detection and quantitation of even smallest amounts (LOD: 0.5 ppm). The minimal sample amount of only a few milligrams, as well as simple and fast performance predestine this method for daily laboratory routine (**paper 1**). Among plant terpenoids, the monoterpene *R*-(+)-limonene is very widespread. Thus, it is not only found in citrus oils but also of in caraway oil, where its proportion amounts to almost 50%. To investigate the storage stability, *R*-(+)-limonene, *S*-(+)-carvone, different caraway oils, and the corresponding caraway seeds were stored in desiccators at 25 °C and 40 °C for eighteen months (**paper 2**). The samples were analyzed monthly by GC/MS and GC/FID, as well as HPLC/DAD-MS/MS. This showed that the comparison of seed, isolated essential oil, and pure substance, which

had not been considered in storage studies so far, was of extraordinary importance. Here, both the plant matrix and the essential oil had a protective effect on individual terpenes and delayed their degradation (**paper 2**). Further, a clear difference between photo-oxidation and autoxidation was observed. Light-induced oxidation of terpenes primarily resulted in the formation of hydroperoxides, whereas autoxidation led to a variety of compounds such as alcohols, ketones, and epoxides. Thus, the secondary products can serve as specific markers for conclusions about the preload and quality of essential oils. In the study presented in **paper 3**, further photo-oxidation experiments were conducted with β-pinene, *R*-(+)-limonene, and γ-terpinene, with added furocoumarins. Furocoumarins can absorb UV-A light in the range of 320 – 380 nm and enter an energetically excited state. This energy difference between the ground state and excited state can be dissipated again by the emission of fluorescent and phosphorescent light. In this process, short-wave energy-rich UV light is converted into lower-energy visible light (bathochromic shift). For this reason, the UV light-induced degradation of the terpenes β-pinene, *R*-(+)-limonene, and γ-terpinene could be significantly reduced by adding 5% each of xanthotoxin, bergapten, bergaptol, and bergamottin. The effect of adding bergaptol was most pronounced in the photo-oxidation of γ-terpinene (**paper 3**). Consequently, in citrus essential oils from which the natural furocoumarins had been previously removed, irradiation with UV light resulted in a strong degradation of the terpenes. This process could be markedly reduced by the re-addition of 5% of the previously removed plant-specific furocoumarins (**paper 4**).

In summary, it can be concluded that not only the plant matrix and the essential oil as a multicomponent mixture but also potential interactions with other substances forming part of the essential oil such as furocoumarins may significantly slow down the oxidation of terpenoids.

7 ZUSAMMENFASSUNG

Ätherische Öle gehören zu den sekundären Pflanzenstoffen, wobei Terpenoide und Phenylpropanoide mengenmäßig zu den Hauptbestandteilen zählen. Aufgrund ihres lipophilen Charakters und ihrer hohen Flüchtigkeit werden diese überwiegend mittels Wasserdampfdestillation gewonnen. Eine Ausnahme bilden ätherische Zitrusöle (Agrumenöle), die in der Regel mittels Pressverfahren aus den Schalen gewonnen werden, wobei auch weniger flüchtige Komponenten wie z.B. Cumarine und Furocumarine in die Ölphase übergehen. Aufgrund ihrer geruchs- und geschmacksgebenden Eigenschaften finden ätherische Öle Anwendung in der Lebensmittel-, Getränke- und Kosmetikindustrie. Darüber hinaus werden sie aufgrund zahlreicher pharmakologischer Eigenschaften in der Phytotherapie sowie in der Aromatherapie eingesetzt. Allerdings sind die meisten ätherischen Öle in Gegenwart von Luftsauerstoff, Licht und bei erhöhten Temperaturen sehr anfällig für Oxidations-, Polymerisations-, Dehydrierungs- und Isomerisierungsreaktionen. Die damit einhergehenden organoleptischen Veränderungen führen meist zu einer deutlichen Qualitätsminderung. Ein weiteres Problem stellt die Bildung von Terpenhydroperoxiden dar, da diese im Verdacht stehen, bei 1-3% der europäischen Bevölkerung bei Hautkontakt Unverträglichkeiten wie Rötungen und Juckreiz auszulösen. Die Erfassung derartiger chemischer Veränderungen ist fester Bestandteil der Qualitätskontrolle, und diese sind durch geeignete Herstellungs-, Transport- und Lagerbedingungen zu minimieren. Die Analytik der ätherischen Öle findet aufgrund ihrer Flüchtigkeit vorwiegend mittels Gaschromatographie statt. Die Detektion von Hydroperoxiden stellt aufgrund ihrer Instabilität jedoch erhebliche Anforderungen an die herkömmlichen Analysemethoden. Deshalb wurde in der in **Paper 1** vorgestellten Studie eine neue spektrophotometrische Methode zur Detektion und Quantifizierung von Peroxiden und Hydroperoxiden in Terpenen und ätherischen Ölen entwickelt. Die Oxidation von *N*-*N*-Dimethyl-*p*-phenylendiamin durch Peroxide zu einem intensiv rot gefärbten Kation (Wursters Rot) ermöglichte die kolorimetrische Detektion und Quantifizierung selbst kleinster Mengen (LOD 0,5 ppm). Die geringe Probenmenge von nur wenigen Milligramm sowie die einfache und schnelle Durchführung prädestinieren diese Methode für die tägliche Laborroutine (**Paper 1**).

Unter den pflanzlichen Terpenoiden ist das Monoterpen *R*-(+)-Limonen sehr weit verbreitet. So findet es sich nicht nur in Zitrusölen, sondern auch mit einem Anteil von

knapp 50% in Kümmelöl. Zur Untersuchung der Lagerstabilität wurden *R*-(+)-Limonen, *S*-(+)-Carvon, verschiedene Kümmelöle und die entsprechende Kümmelsaat achtzehn Monate lang bei 25 °C und 40 °C in Exsikkatoren gelagert (**Paper 2**). Mittels GC/MS und GC/FID sowie HPLC/DAD-MS/MS wurden die Proben monatlich analysiert. Hierbei zeigte sich, dass der bisher in Lagerstudien nicht berücksichtige Vergleich von Saat, ätherischem Öl und Reinstoff von außerordentlicher Bedeutung ist. Dabei wirkten sich sowohl die Pflanzenmatrix als auch das ätherische Öl schützend auf die einzelnen Terpene aus und verzögerten deren Abbau (**Paper 2**). Dabei zeigte sich zudem ein deutlicher Unterschied zwischen der Photooxidation und der hier ablaufenden Autoxidation. Bei der lichtinduzierten Oxidation von Terpenen werden primär Hydroperoxide gebildet, während die Autoxidation zu einer Vielzahl von Verbindungen wie Alkoholen, Ketonen und Epoxiden führt. So können die Folgeprodukte als spezifische Marker zur Bewertung der Vorbelastung und Qualität eines ätherischen Öles herangezogen werden. In der in **Paper 3** dargestellten Studie wurden weitere Photooxidationsversuche mit β-Pinen, *R*-(+)-Limonen und γ-Terpinen durchgeführt, denen zuvor Furocumarine zudotiert wurden. Furocumarine können UV-A Licht im Bereich 320 – 380 nm absorbieren und gehen dabei in einen energetisch angeregten Zustand über. Die Energiedifferenz zwischen Grundzustand und angeregtem Zustand kann durch Emission von Fluoreszenz- und Phosphoreszenzlicht dissipiert werden. Hierbei wird energiereiches kurzwelliges UV-Licht in energieärmeres, sichtbares Licht umgewandelt (bathochromer Shift). Aus diesem Grund konnte der UV-Licht induzierte Abbau der Terpene β-Pinen, *R*-(+)-Limonen und γ-Terpinen durch die Zugabe von je 5% Xanthotoxin, Bergapten, Bergaptol und Bergamottin erheblich reduziert werden. Der deutlichste Effekt wurde bei Zugabe von Bergaptol zu γ-Terpinen beobachtet (**Paper 3**). Folgerichtig kam es bei ätherischen Zitrusölen, aus denen die natürlichen Furocumarine zuvor entfernt wurden, durch Bestrahlung mit UV-Licht zu einem starken Abbau der Terpene. Dieser Prozess konnte durch erneute Zugabe von 5% der zuvor entfernten pflanzenspezifischen Furocumarine deutlich verringert werden (**Paper 4**).

Zusammenfassend lässt sich feststellen, dass nicht nur die pflanzliche Matrix und das ätherische Öl als Mehrkomponentengemisch, sondern auch die Wechselwirkung mit anderen, im ätherischen Öle enthaltenen Substanzen wie Furocumarinen die Oxidation von Terpenoiden signifikant verlangsamen kann.

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