Sesquiterpenes and sesquiterpenoid derivatives in food aromas: Physiological derivates and structure-activity relationships in the modulation of $GABA_A$ and glycine receptors

Sesquiterpen- und Sesquiterpenoidderivate in Lebensmittelaromen: Physiologische Derivate und Struktur-Wirkungsbeziehungen in der Modulation von $GABA_A$- und Glycin-Rezeptoren

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List of Abbreviations

A

AAS *Atomic absorption spectroscopy*
Acetyl-CoA *Acetyl-coenzyme A*
amu *Atomic mass units*
aSAFE *Automated solvent-assisted flavour evaporation*
ATP *Adenosine Triphosphate*

C

CCC *Countercurrent chromatography*
CI *Chemical ionization*
CNS *Central nervous system*

D

DAD *Diode array detector*
DMAPP *Dimethylallyl pyrophosphate*

E

EI *Electron ionization*
EO *Essential oil*
ESI *Electrospray ionization*

F

FPP *Farnesyl diphosphate*

G

GABA *γ-Aminobutanoic acid*
GABAAR *$GABA_A$ receptor*
GC *Gas chromatography*
GGPP *Geranyl geranyl diphosphate*
GIT *Gastrointestinal tract*
GPCR *g-protein coupled receptor*
GPP *Geranyl diphosphate*

H

HPLC *High performance liquid chromatography*
HPTLC *High performance thin layer chromatography*

I

IPP *Isopentenyl pyrophosphate*
IR *Infrared spectroscopy*

M

m/z *Mass-to-charge ratio*
MALDI *Matrix assisted laser desorption/ionization*
MEP *Methylerythritol phosphate*
MS *Mass spectrometry*
MVA *Mevalonate*

N

NMR *Nuclear magnetic resonance*

O

OR *Olfactory receptor*

S

SAFE *Solvent assisted flavour evaporation*
SQTs *Sesquiterpenes and sesquiterpenoids*

T

TLC *Thin layer chromatography*
TOF *Time-of-flight*

Abstract

Sesquiterpenes and sesquiterpenoids (SQTs) are a frequently occurring natural substance class with diverse chemical structures and represent an important compound class in many essential oils. Only a few of these substances could be synthesized or isolated so far, so that biochemical and physiological studies of individual components are rare. Synergistic effects based on the interaction of two or more molecules are even less studied. Since SQTs are also components of foods, it is important to obtain knowledge about their metabolization. Furthermore, these substances may modulate the $GABA_A$ receptor, which is the most important inhibitory receptor in the central nervous system. This receptor mediates the calming, anxiety-relieving or sedative properties of some natural substance mixtures and extracts.

In this work, five plants were selected and first their SQTs profiles were elucidated by gas chromatography coupled with mass spectrometry. A total of 52 SQTs could be identified and also quantified above a defined threshold concentration. The results complement previously reported findings with the addition of quantitative values. Subsequently, the focus of this work was on the isolation of SQTs, as only a few of the identified structures were commercially available. Here, the sesquiterpenoids α-bisabolol oxide A, spathulenol, α-bisabolol oxide B and α-bisabolone oxide A were obtained from dried chamomile flowers (*Matricaria chamomilla* L.) by combining different chromatographic methods, in particular countercurrent chromatography. The plant was selected due to the overall high SQTs content in the volatile fraction and the promising structures for physiological studies. In addition, other extraction and distillation methods as well as preparative gas chromatography were used to produce defined extracts and to isolate further substances. Six molecules were selected by using these techniques, and additionally other commercially available SQTs. Then, their possible metabolization in the human body was investigated using an artificial *in vitro* digestion system. It could be shown that especially the acidic environment during the stomach phase is responsible for metabolic changes. Overall, however, it was found that the selected SQTs remain structurally unchanged or are only converted with low conversion rates. These findings were used for $GABA_A$ receptor studies in collaboration with the working group of Prof. Villmann, University of Würzburg. Along the 11 structures for these studies, two of the

selected substances were humulol and caryolanol, which are transformed from α-humulene and β-caryophyllene during metabolization in the human body. With $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_4\beta_3\delta$, as well as the $\alpha_6\beta_3\delta$ subtype, different receptor configurations were chosen for these investigations. Both positive and negative allosteric modulators could be found, but most SQTs are to be considered negative modulators on these subtypes. When measuring hippocampal neurons, only the sesquiterpenoid guaiol could be confirmed as a positive modulator from the tested structures.

Zusammenfassung

Sesquiterpene und Sesquiterpenoide (SQTs) sind häufig vorkommende und strukturell vielfältige Naturstoffe und stellen bei vielen ätherischen Ölen einen wichtigen Hauptbestandteil dar. Nur ein Bruchteil dieser Substanzen konnte bisher synthetisiert oder isoliert werden, so dass biochemische und physiologische Untersuchungen von Einzelkomponenten bisher selten sind. Noch weniger untersucht sind synergistische Effekte, die auf dem Zusammenwirken von zwei oder mehreren Molekülen basieren. Da SQTs auch Bestandteile der Nahrung sind, ist es von Bedeutung Kenntnisse über deren Metabolisierung zu erhalten. Darüber hinaus können diese Stoffe den $GABA_A$-Rezeptor, den wichtigsten inhibitorischen Rezeptor im zentralen Nervensystem modulieren und so für die beruhigenden, angstlösenden oder sedierenden Eigenschaften von einigen Naturstoffgemischen und Extrakten verantwortlich sein.

Im Rahmen dieser Arbeit wurden fünf Pflanzen ausgewählt und zunächst deren SQTs-Profile durch gaschromatographisch-massenspektrometrische Untersuchungen aufgeklärt. Insgesamt konnten 52 SQTs identifiziert und ab einer definierten Schwellenkonzentration auch quantifiziert werden. Die Ergebnisse bestätigten bereits zuvor berichtete Ergebnisse, und komplementieren diese durch die quantitativen Werte. Anschließend lag der Fokus dieser Arbeit auf der Isolierung von SQTs, da nur wenige der identifizierten Strukturen kommerziell verfügbar waren. Hierbei konnten durch die Kombinationen unterschiedlicher chromatographischer Verfahren, insbesondere der Gegenstromverteilungschromatographie, die Sesquiterpenoide α-Bisabolol oxid A, Spathulenol, α-Bisabolol oxid B und α-Bisabolon oxid A aus getrockneten Kamillenblüten (*Matricaria chamomilla* L.) gewonnen werden. Die Pflanze wurde aufgrund des insgesamt hohen SQTs Gehalts in der volatilen Fraktion und der für physiologische Untersuchungen interessanten Strukturen ausgewählt. Darüber hinaus wurden weitere Extraktions- und Destillationsverfahren, sowie die präparative Gaschromatographie verwendet, um definierte Extrakte herzustellen sowie weitere Stoffe zu isolieren. Aus diesen und weiteren kommerziell erhältlichen SQTs wurden sechs unterschiedliche Moleküle ausgewählt und deren mögliche Metabolisierung im menschlichen Körper durch ein künstliches *in vitro* Verdauungssystem untersucht. Dabei konnte gezeigt werden, dass insbesondere das saure Milieu während der Magen-Phase verantwortlich für

Stoffänderungen ist. Insgesamt ließ sich aber feststellen, dass die ausgewählten SQTs größtenteils unverändert, bzw. nur mit niedrigen Konversionsraten transformiert werden. Diese Erkenntnisse wurden für $GABA_A$ Rezeptorstudien in Zusammenarbeit mit der Arbeitsgruppe Prof. Villmann, Universität Würzburg, herangezogen, denn bei den für diese Untersuchungen ausgewählten 11 Strukturen wurden mit Humulol und Caryolanol auch Substanzen gewählt, die erst bei der Verstoffwechselung im Körper aus α-Humulen und β-Caryophyllen entstehen. Es wurden mit $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_4\beta_3\delta$, sowie der $\alpha_6\beta_3\delta$ Variante verschiedene Rezeptorkonfigurationen für diese Untersuchungen gewählt. Es konnten sowohl positive als auch negative allosterische Modulatoren gefunden werden, die meisten SQTs sind auf den getesteten Subtypen dabei aber als negative Modulatoren zu betrachten. Bei der Messung an hippocampalen Neuronen konnte nur das Sesquiterpenoid Guaiol von den getesteten Strukturen als positiver Modulator bestätigt werden.

1. Introduction

1.1. The Kingdom of Plants and its naturally occurring Compounds

With the emergence of the first plant species about 2.1 billion years ago, and the appearance of the first land plants about 470 million years ago, these manifold manifestations of life are historically clearly ahead of humankind [1]. Plants are one of the five main groups of eukaryotes and until today around 290.000 species have been described. Amongst these, the flowering plants (angiosperms) are the most species-rich subgroup, with around 240.000 species. Besides the fertility-driving factor insect pollination and the diversity of the vegetative morphology, it is assumed, that also the huge number of secondary metabolites are a reason for the species' richness.

The secondary metabolism, or specialized metabolism, can be distinguished from the primary metabolism, which shows great similarities in plants, animals and humans [2]. It comprises all processes and substances, which are essentially involved in growth and development of individuals. This includes for instance the use of amino acids, carbohydrates and lipids as basic building blocks. Also, regardless if in single-cell organisms or in humans, the genetic code for the assignment of nucleotide triplets to amino acids, but also the use of phosphates e.g., adenosine triphosphate (ATP) for energy storage and transfer, is universal. Any non-essential processes and substances beyond the primary metabolism belong to the secondary metabolism. The unambiguous assignment however is often fluid. The secondary metabolism concerns the interactions of individuals with the environment, and the respective metabolites are synthesized from special substances from the primary metabolism. Hence, all substances fulfill a physiological purpose, in plants for example as a protection against predators, as an antimicrobial barrier or for the sake of communication, e.g., with insects.

Secondary metabolites can be found in all parts of the plant, although they are accumulated in specific plant organs, according to their respective physiological purpose [2]. Vacuoles, plastids or idioblasts serve as deposition sites inside or outside of the plant cells. The ontogenesis and the seasons, amongst others, have a great influence on the qualitative and quantitative composition. Furthermore, there are also abiotic factors such as the temperature,

the soil quality or the amount of rainfall as well as biotic factors such as pests, and all these taken together can influence the composition of secondary plant metabolites [2, 3].

Due to the large number of structural features and combinations, it is difficult to classify secondary metabolites. Nevertheless, there are many similar structures, which can be classified by a common biosynthetic pathway. On the other hand, the structural diversity within a group also means that the function or effects of the individual molecules can vary greatly. Therefore, another type of classification can be made according to the functions and effects e.g., bitter substances, which are characterized by a common bitter taste (c.f. chapter 1.5.1.). An overview of some important secondary plant metabolite classes is shown in Figure 1.

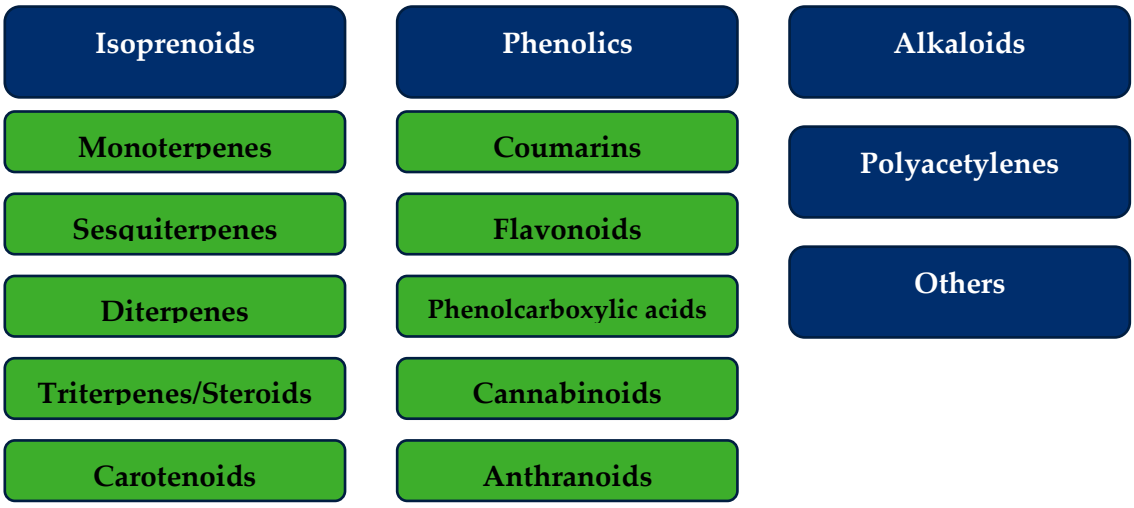


Figure 1: Selection of some important secondary plant metabolites [1, 2, 4].

Another classification of secondary plant metabolites can be made by the differentiation of volatile and non-volatile substances. Volatile substances, which can mostly be characterized by their lipophilicity, can be found in the so-called essential oil (EO) of a plant. According to the definition within the ISO 9235, this EO can be obtained by steam distillation (cf. chapter 1.3.1.), or mechanical squeezing (citrus fruits). EOs predominantly contain volatile and lipophilic secondary metabolites, mainly from the substance classes of phenylpropanoids and terpenes [2]. Apart from EOs, there are several other possibilities to access the secondary plant metabolites, for instance via supercritical fluid extraction with CO_2 or solvent extraction for instance with hexane or dichloromethane. However, in all extraction techniques, also minor

constituents from other secondary plant metabolite substance classes, like flavonoids, sesquiterpene lactones or coumarins, can be co-dissolved.

The medicinal potential of plants has long been known and used in traditional medicine. The wide range of applications of EOs is just one example out of many. The described healing effects of medicinal plants range from anti-inflammatory and antioxidant properties to calming and sedative effects [5-10]. Although the herbal medicine is closely linked to the history of humankind, it is still not fully acknowledged within the evidence-based medicine. However, in their report from 2019, the World Health Organization (WHO) states, that the "Traditional and complementary medicine (T&CM) is an important and often underestimated health resource […]" [11]. It is obvious that due to the large number and diversity of natural products (for instance EOs containing hundreds of compounds), an equally large number of studies are needed to investigate their medicinal potentials objectively and critically. The focus of individual studies should therefore also be based on substance specific investigations, e.g., on isoprenoids.

1.1.1. Isoprenoids

Isoprenoids, also known as terpenes or terpenoids, are one of the most important substance classes of secondary plant metabolites and often represent the main substance class in EOs. With around 35.000 representatives they are also the most diverse and largest group of naturally occurring compounds [2].

Two possible biosynthetic pathways are characterized so far [2, 12]. The first one is called the mevalonate pathway (MVA), where acetyl-coenzyme A (acetyl-CoA) serves as the starting substance, which itself is an important molecule involved in many general cell metabolism processes. Via various intermediate steps, in which different enzymes are involved, the isoprenoid precursors isopentenyl pyrophosphate (IPP) and the isomeric dimethylallyl pyrophosphate (DMAPP) are formed. Besides the MVA pathway, another possibility for the biosynthesis of IPP and DMAPP exist, which has about two decades ago been discovered [13]. Named after the starting substance methylerythritol phosphate (MEP) it is called the MEP or non-mevalonate pathway.

By prenyltransferases, geranyl diphosphate (GPP) is formed from one molecule of each IPP and DMAPP. Farnesyl diphosphate (FPP), geranyl geranyl diphosphate (GGPP) and geranyl farnesyl diphosphate (GFPP) are formed by further addition of IPP building blocks. This biosynthetic pathway is depicted in Figure 2. Thus, all isoprenoids are formally composed of isoprene units and are classified according to their respective number of building blocks. This is also referred to as the so-called biogenetic isoprene rule established by Leopold Ružička in 1953 [14]. Monoterpenes, which originate from GPP, represent the basic unit for the counting method. Sesquiterpenes (sesqui = one and a half) are composed of 15 and diterpenes of 20 carbon atoms. The derivatives of terpenes, for instance formed by oxidation, are called terpenoids.

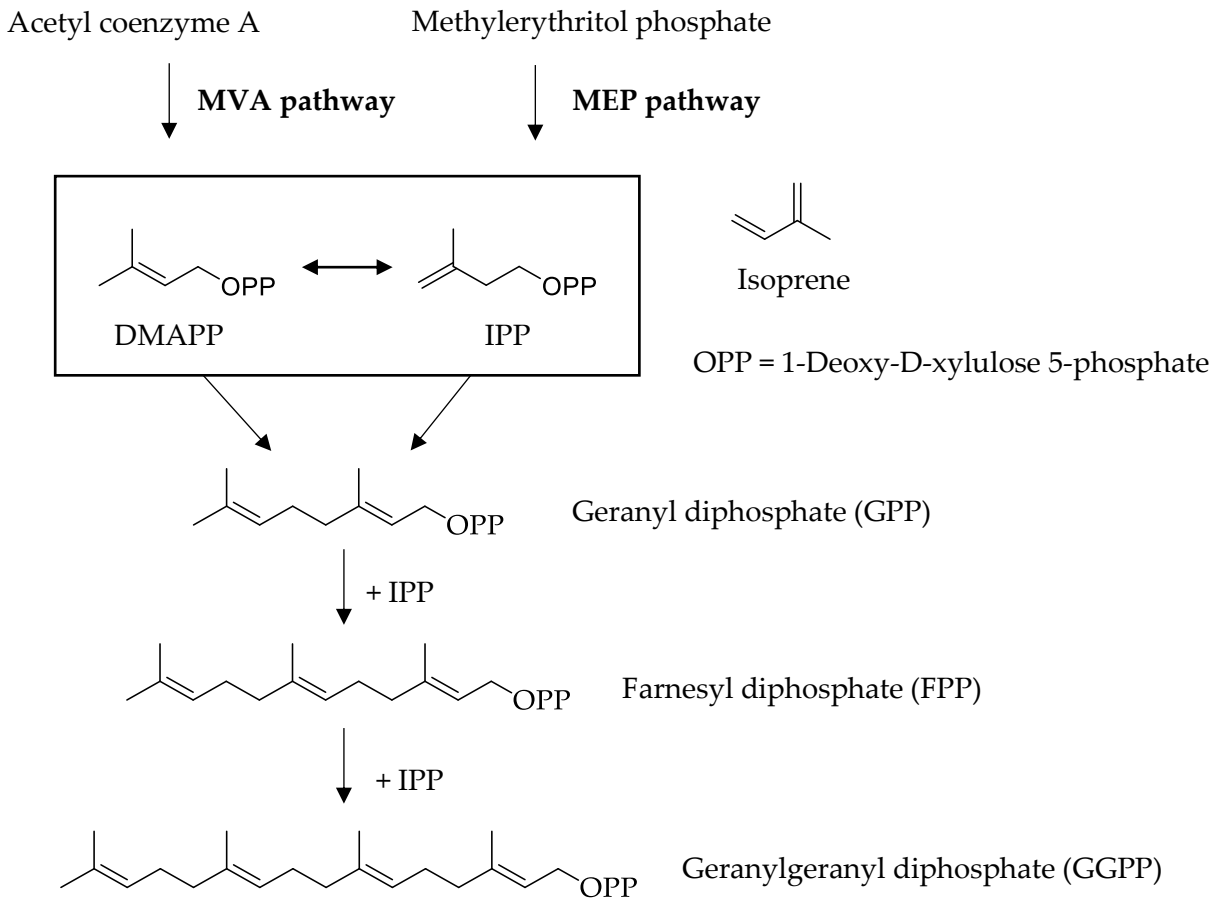


Figure 2: Biosynthesis of isoprenoids. Higher isoprenoids are synthesized by combining the structures depicted here or by the addition of IPP. MVA = mevalonate, MEP = methylerythritol phosphate.

This structural diversity explains the large number of manifold isoprenoid representatives in the plant kingdom. The monoterpenoid menthol, for example, is an important component of peppermint oil [15]. α-Humulene is a frequently occurring sesquiterpene that is found in high concentrations e.g., in hops, and can have a decisive influence on the hop aroma [12]. Larger and even more complex representatives of the isoprenoid family are steroids with the parent molecule cholesterol, from which other steroids are derived [2]. Carotenoids with 40 carbon units (eight isoprene units) are also isoprenoids with β-carotene being the most famous representative [15]. The structures of these representatives are depicted in Figure 3.

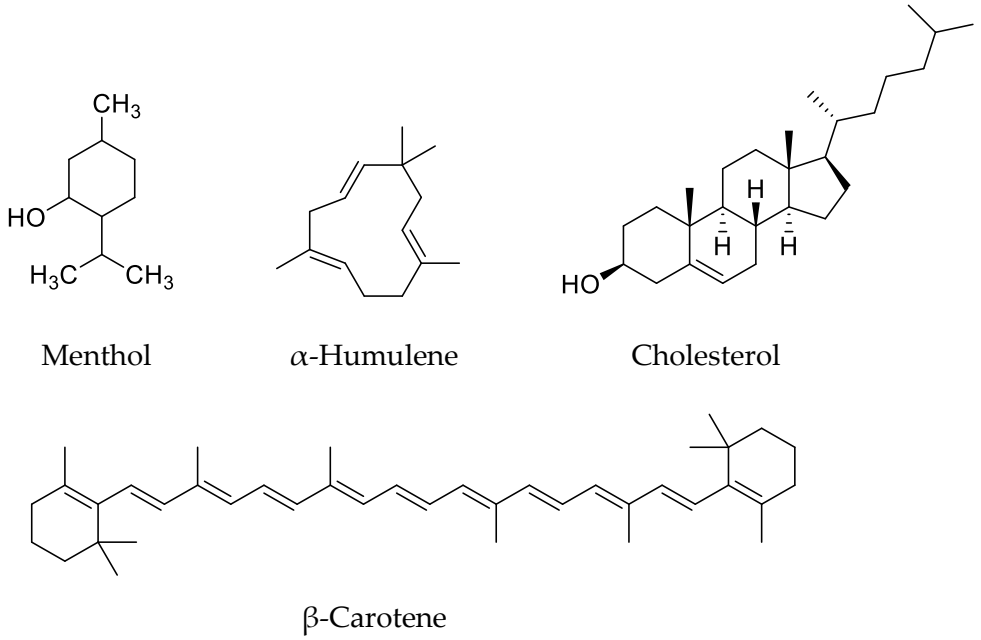


Figure 3: Structures of some important isoprenoid representatives.

Although the biochemical roles of carotenoids and steroids are well understood, the functional role of most other isoprenoids in nature is not yet fully resolved [16]. More research is needed to investigate the effects of plant derived isoprenoids on humans. As main components of EOs, sesquiterpenes and sesquiterpenoids (SQTs) are an important substance class and require more detailed investigations.

1.1.1.1. Sesquiterpenes and Sesquiterpenoids

With over 11000 known compounds and more than 100 different carbon skeletons, the class of SQTs represent the largest subgroup within the isoprenoids [2, 17-19]. Commonly, they are classified according to their number of carbon rings, which range from acyclic to tetracyclic

systems. All SQTs can be derived from the formerly mentioned precursor FPP and therefore consist of three isoprene units or 15 carbon atoms (Figure 2). In plants, the MVA pathway is the main biosynthetic route for the production of SQTs, which takes place in the cell's cytosol [12, 13]. Plant-specific enzymes, like the sesquiterpene synthases, often produce a variety of different SQTs, which is caused by the fact that the intermediates are structurally well stabilized [12, 20]. Many plants are characterized by a high concentration of SQTs in the volatile fraction. Five examples of these foods will be introduced in the next chapter.

1.2. Foods with high Contents of SQTs

SQTs are important components of EOs but also occur in fruits, vegetables, herbs and spices [15]. In this chapters, five edible plants are introduced, which are characterized by a high content of SQTs on the one side. On the other side they show sedative, anxiolytic, sleep-inducing or calming effects. Thus, some substances or combinations of them might be active in the modulation of the human $GABA_A$ receptor (cf. Chapter 1.5.2.).

1.2.1. Hop

Hop (*Humulus lupulus* L.) is a perennial climbing plant of the Cannabaceae family, where also cannabis is a member [1, 15, 21]. It is mainly cultivated in moderate climate regions, with Germany and USA being the main cultivation countries [22]. In the industrial farming, only female plants are cultivated, whose inflorescences form the so-called hop cones, which are harvested in late summer to autumn. The bitter substances and the EO are produced and stored in the lupin glands and are particularly important for the brewing industry. The amount of EO in dried hop cones can reach up to 3 % [12, 22]. More than 450 volatile substances in the EO of hop have already been identified so far, although the total number is estimated to be much higher. The most important compounds can be assigned to the substance classes of mono- and sesquiterpenes, including β-myrcene, α-humulene and β-caryophyllene [12, 23, 24].

Figure 4: Dried hop cones from Humulus lupulus L.

Hop has long been used in the traditional medicine, for example against insomnia, anxiety or nervousness [5]. There are different types of application and administration forms, including the consumption of tea or the application of sleeping pillows or tinctures. Current research focuses on the antioxidant properties, antimicrobial activities and the sedative effects of hops amongst others [25].

1.2.2. Lavender

True lavender (*Lavandula angustifolia*) is a perennial plant of the Lamiaceae family, which is native and being cultivated in the Mediterranean region [5, 21, 26]. The lower parts of the plant are often lignified, whereas the upper parts bear silver-green leaves. The flowers are of pale violet color and are harvested in the summer months July and August. The plant is part of the Mediterranean cuisine, whereby the cultivation is mainly used to produce EO, which is extracted from the flowers by means of steam distillation. Besides true lavender (*Lavandula angustifolia*), other subspecies, cultivars and hybrids exist.

Figure 5: Dried lavender flowers from lavandula angustifolia.

Flavonoids, phytosterols and anthocyanins are important constituents of true lavender, together with the EO that can reach up to 3% in content [5, 26]. More than 300 volatile substances have been detected so far. The main components include the monoterpenoids linalool, linalyl acetate, terpinen-4-ol and camphor but also sesquiterpenes like caryophyllene [12, 26, 27].

Lavender has been used in the traditional medicine since the ancient Romans and Greeks. The term is derived from the Latin word “lavare”, which means “to wash”, since it was an important component of the Roman Bath [5, 26]. Furthermore, the herb is valued for its calming effects and is also used as an insect repellent. Typical forms of administration are teas and tinctures as well as inhalation or direct application of the EOs to the skin.

1.2.3. Lemon balm

Besides Lavender, lemon balm (*Melissa officinalis* L.) is another member of the Lamiaceae family. The perennial shrub, which is native to the Mediterranean and western Asia, can grow to a height of 80-100 cm [28, 29]. Today, it is mainly cultivated in Europe, North Africa and North America [2]. The flowering period ranges from June to August. The name of the herb originates from the lemon-like scent of the leaves. They can grow up to 8 cm long and 3 cm wide, and their extracts are commonly used in drug applications [21].

Figure 6: Fresh lemon balm leaves (Melissa officinalis L.).

Important secondary plant constituents are flavonoids, phenolic compounds and the EO (max. content ~0.3 %) [28-30]. Important components of the EO are the monoterpenoids citral and citronellal as well as the sesquiterpene β-caryophyllene [31].

In the traditional medicine, lemon balm is known for its calming and antispasmodic effects [5]. It is also used externally for the healing of cold sores. Antibacterial and antioxidant properties amongst other effects were detected recently [28, 30, 31].

1.2.4. Chamomile

True chamomile (*Matricaria chamomilla* L. syn. *Chamomilla recutita* (L.) RAUSCHERT) is one of the most important medicinal plants and has been known in the traditional medicine since ancient times [32]. The annual plant from the family of Asteraceae originates from southern and eastern Europe as well as the middle East and is now spread worldwide and mainly cultivated in Argentina, Egypt and Hungary [2, 21, 33]. It can reach heights of up to 80 cm and forms yellow flower heads surrounded by white ray florets. The characteristic blue EO can be extracted from the flowers by steam distillation. However, this color is only formed due to the

Figure 7: Dried chamomile flower heads from Matricaria chamomilla L.

processing, where the sesquiterpene lactone matricin is transformed into chamazulene, which is the artefact responsible for the blue color.

Important constituents of the plant are flavonoids, coumarins, polyacetylenes and the EO (max. content ~ 1.5%) [21, 33]. It mainly contains sesquiterpenes and sesquiterpenoids, such as (*E*)-β-farnesene, α-bisabolol and the α-bisabolol oxides A and B [34, 35]. Although the qualitative composition is consistent, there can be great differences in the quantitative composition of the EO. For example, the α-bisabolol content in various European countries can vary between 0.1 % and 44.2 % [34].

In the traditional medicine, the plant's antispasmodic, wound-healing, antifungal and calming effects are known [2, 5, 32]. Both internal and external applications are in use. Most commonly, dried flower heads are used for preparations such as teas, extracts or tinctures.

1.2.5. Basil

Basil (*Ocimum basilicum* L.) is an annual herb from the Lamiaceae family. Due to complex taxonomy, the exact place of origin of the plant is not known [21]. Today, however, the plant is cultivated mainly in the Mediterranean region and in India. The plant grows to a height of up to 50 cm and produces white flowers at the tips of its branches. Different subspecies and varieties exist, like Thai basil (var. thyrsiflorum) or lemon basil (var. citriodora) [36]. Some of the cultivars strongly differ in their secondary plant metabolite profile. For example, the green and purple cultivars can be distinguished not only morphologically, but also by their chemical composition. In a comparative study with 27 cultivars, overall, 87 volatile substances were detected, some of them were even specific to one cultivar [37].

Figure 8: Fresh leaves of basil (Ocimum basilicum L.)

Besides polyphenols and flavonoids, the plant contains a relevant proportion of EO (content ~0.7%). The main components are linalool, 1,8-cineole, eugenol and the sesquiterpenes epi-α-cadinol and α-bergamotene. In the traditional medicine, the herb is described as carminative

and anti-spasmodic [21, 38]. Furthermore, the herb is very popular in the Mediterranean cuisine.

1.3. Isolation and Characterization of Natural Products

As discussed in the previous chapters, plants synthesize a vast number of various secondary plant metabolites. To properly identify and consequently characterize substance classes or, beyond that, individual molecules, it is necessary to separate or isolate them from complex matrices and mixtures. In the analytical and preparative (bio-)organic chemistry, a large number of techniques and methods is available to overcome this challenge. Some of these will be presented in more detail in the following chapters. Thereby, the selection will cover techniques and methods, which are suitable for isoprenoids, in particular for SQTs.

1.3.1. Matrix Elimination and Enrichment of Secondary Plant Metabolites

When investigating isoprenoids in plant materials, one initially encounters a variety of heterogeneous matrices. The flowers, leaves, stems etc. have different shapes, sizes, or water contents. Therefore, a first step is to homogenize the plant material to produce a homogeneous and representative sample [39]. This can be done, e.g., by crushing or cutting the plant material. Afterwards, the enrichment of the target components can be performed. During this process, the analytes often pass into a different phase than the plant's matrix [40].

Many processes are based on the use of an organic solvent, such as the classic extraction techniques maceration, decoction, percolation, Soxhlet and reflux extraction [41]. The choice of the solvent should thereby already be adapted according to the next preparative or analytical steps. In addition to the conventional techniques, which often require long extraction times and much solvent, more advanced techniques have been developed in the past decades [40]. These include microwave-assisted extraction, ultrasound-assisted extraction, enzyme-assisted extraction, pulsed electric field-assisted extraction, pressurized liquid extraction, pulsed electric field extraction and supercritical fluid extraction (Figure 10) [40-43]. The advantages are faster extraction times, lower solvent consumption and, most importantly, an improved extraction yield [44].

Techniques for enriching target compounds also include distillation processes. As already mentioned in chapter 1.1., steam distillation plays a special role, since it is used to isolate the EO of a plant. Three subtypes of this distillation technique exist, which are water distillation, water and steam distillation and direct steam distillation [42]. Since high temperatures are required in each case, this technique is not suitable for thermolabile substances. To enable distillation of thermolabile substances, comprising many odor-active compounds, solvent assisted flavour evaporation (SAFE) was developed [45]. A special glass apparatus is used in this technique (Figure 9). Under high vacuum, volatile substances can be quickly separated from the matrix without the need for high temperatures. This reduces the formation of artefacts. Furthermore, SAFE also achieves higher yields. Recently, an automated SAFE (aSAFE) approach with even higher yields was introduced [46]. Further distillation techniques used for the enrichment of isoprenoids are bulb-to-bulb distillation and rotary evaporators for the removal of the solvent matrix.

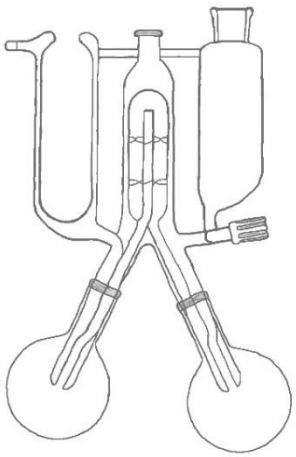


Figure 9: Glass apparatus used in the solvent-assisted flavour evaporation (SAFE) technique.

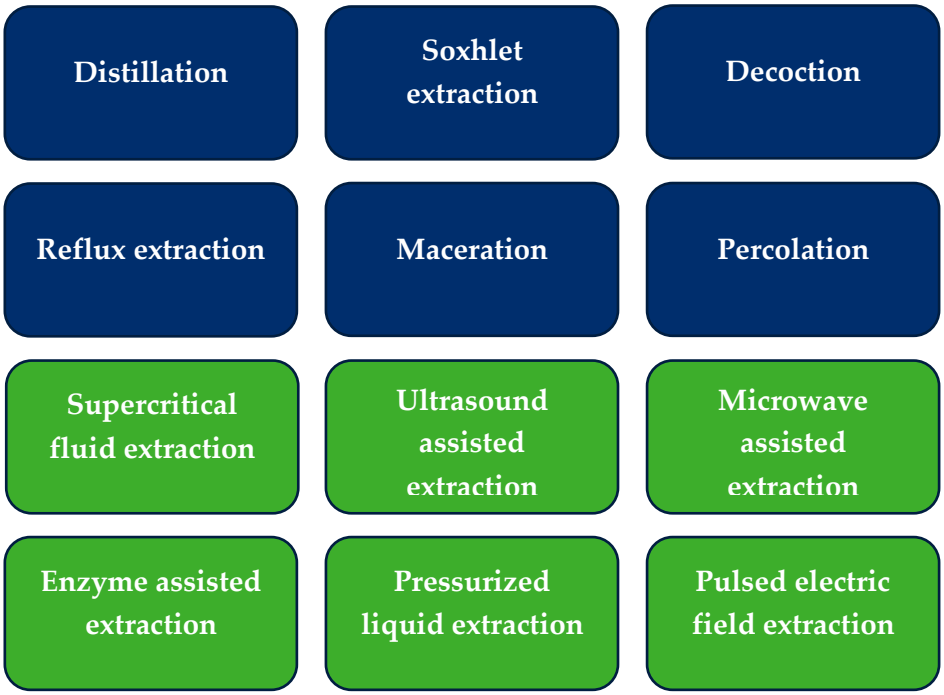


Figure 10: List of extraction and enrichment methods with conventional (blue) and contemporary methods (green).

Various techniques for the enrichment can be combined according to the requirements of the target compounds. However, it is crucial to tailor the processes in such way that allows the direct application of extracts for consecutive separation steps.

1.3.2. Physico-Chemical Separation Techniques

Extracts from natural products are enriched with respect to specific target components, thus mostly eliminating interfering matrix. However, such extracts may still contain too many substances for unambiguous analyses so that application of the extracts to further, potentially consecutive separation processes may be required. These processes can be classified either as separation steps with or without substance conversion [40]. Separation techniques with substance conversion include e.g., precipitation or electrolytic reactions. Separations without substance conversion can be classified into four separation principles, i.e., distribution, particle charge, particle mass and vapor pressure [40]. Separations by use of vapor pressure also include the distillation techniques described in chapter 1.3.1. Separations due to particle charge are indispensable for mass spectrometry (MS) and are described in more detail in chapter 1.3.3. However, the most important physico-chemical separation technique for naturally occurring substances is chromatography [40, 41, 47].

Chromatographic methods are based on the distribution of substances between a stationary and a mobile phase. The separation principles are based on adsorption processes, distribution, ion exchange and gel permeation. There are different principles to classify chromatographic techniques and methods, a concept proposed in literature is given in Figure 11 [40].

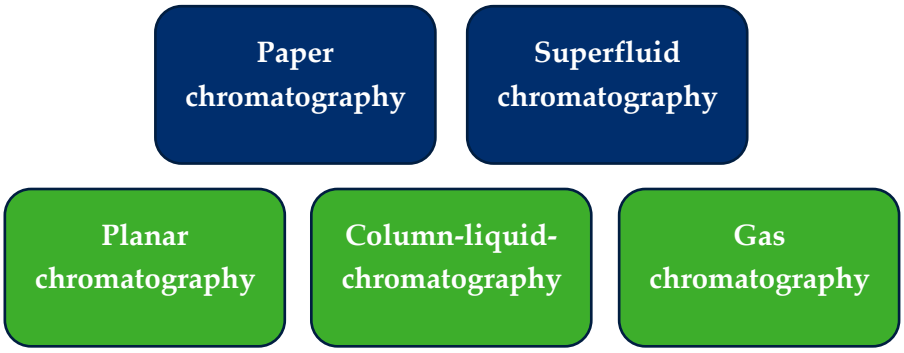


Figure 11: Classification of chromatographic techniques according to [40]. The methods depicted in green are discussed within the scope of this work.

1.3.2.1. Planar Chromatography

In planar or thin layer chromatography (TLC), the mobile phase consists of a solvent and the stationary phase is a sorbent [39, 40, 47]. The most common materials used are silica gel or alumina (aluminum oxide). The plates on which the sorbent is applied with a layer thickness of 0.1-0.25 mm are made of glass, aluminum, or plastic. Cellulose can also be used, therefore the former method paper chromatography is now only rarely used [40]. The substances in the extract are distributed according to their individual solubilities and adsorption properties. Thus, each substance has a characteristic value (the so-called retention factor). The chromatography is carried out in such a way, that the extract is applied to the plate. Then, the solvent runs over the plate and distributes the substances (Figure 12). Common chemical solvents or mixtures of them, such as hexane, methanol or toluene, are used as the mobile phase. High performance thin layer chromatography (HPTLC) is a further instrumental development, which can be automated and offers the possibility of quantitative evaluations [40]. Despite the establishment of more sophisticated methods, TLC is still an important method for the analysis of plant constituents.

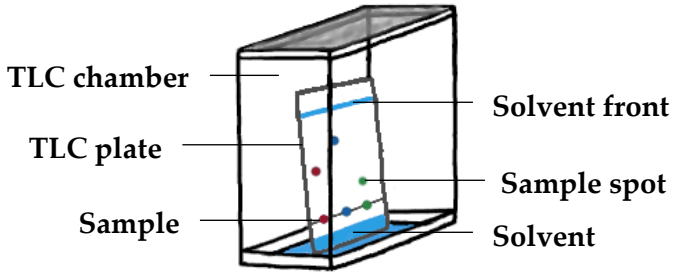


Figure 12: Principle of TLC. The substances are deposited on a plate and are distributed specifically by the mobile phase.

1.3.2.2. Column-liquid Chromatography

In contrast to planar chromatography, columns are being used in the separation techniques presented in this chapter. At first, a distinction can be made whether the separation takes place at normal pressure or at elevated and additionally imposed pressures [40].

The conventional method (normal pressure) is still used today, as it is very easy to apply, since no special equipment is needed. In this process, a glass column is filled with silica gel or, more rarely, alumina. The sample material is applied to the top of the column and the compounds are separated by molecule-specific hydrogen bonds or dipole-dipole interactions (Figure 13) [41]. The mobile phase consists of single solvents or mixtures of them. The eluate is collected in fractions at the end of the column. The disadvantages of this method are slow separations and the possible irreversible adsorption of the dissolved substances [41].

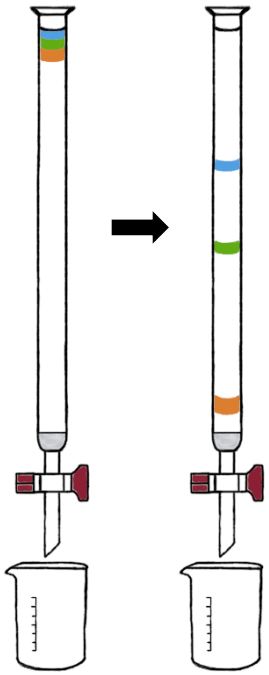


Figure 13: Process of a column chromatography performed at lab scale to separate a mixture of three substances (depicted in blue, green and orange).

High performance liquid chromatography (HPLC) is a technical development of the conventional method that works at high pressures (≤ 400 bar) [39]. The four instrumental components of HPLC are the pump, the injection system, the separation column and the detector (Figure 14) [40].

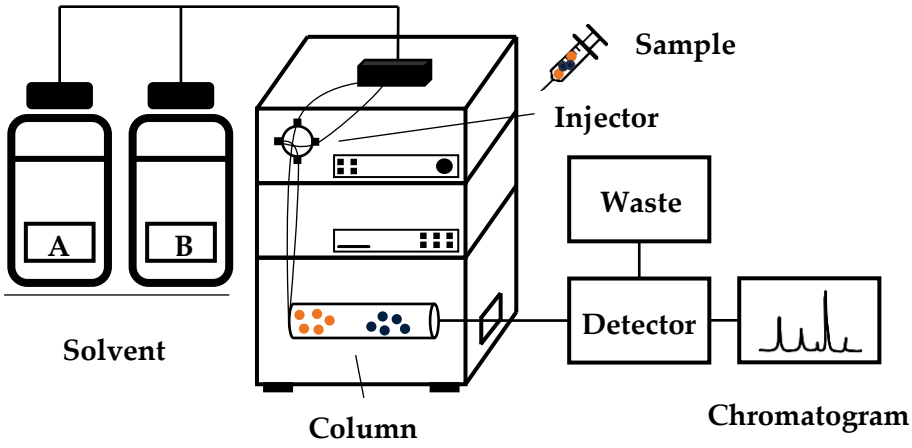


Figure 14: Structural set-up of a HPLC system. The separated sample is analyzed in the detector, resulting in a chromatogram.

HPLC enables both analytical and preparative separations of almost all substance classes. However, the condition for a successful separation is the dissolvability of the sample in the mobile phase [39]. Several separation procedures for the stationary phase exist. The most frequently used method is adsorption chromatography, which can be classified into normal and reversed-phase HPLC [40]. If the stationary phase is polar and the mobile phase is non-polar, this is referred to as a normal phase system. However, the most important method is reversed-phase HPLC, in which the stationary phase is non-polar and the mobile phase is polar [40]. Modified silica gel, often with alkyl moieties such as octadecylsilane (C-18) or octylsilane (C-8), is used as column material [39]. Water as well as water-methanol or acetonitrile-water mixtures are used as the mobile phase. For structural elucidations of plant derived compounds, many hyphenated techniques exist (cf. chapter 1.3.3.).

A similar principle, but with a liquid as the stationary phase, is used in countercurrent chromatography (CCC) [47, 48]. The separation of the substances is based on the molecule-specific distribution between two immiscible phases. Both the stationary and the mobile phase are part of a two-phase solvent system. One phase is held stationary by a planetary movement around two axes of a coil, which is surrounded by a tube. The other phase is pumped through the column and is fractionated at the end or directly monitored by a detector (Figure 15).

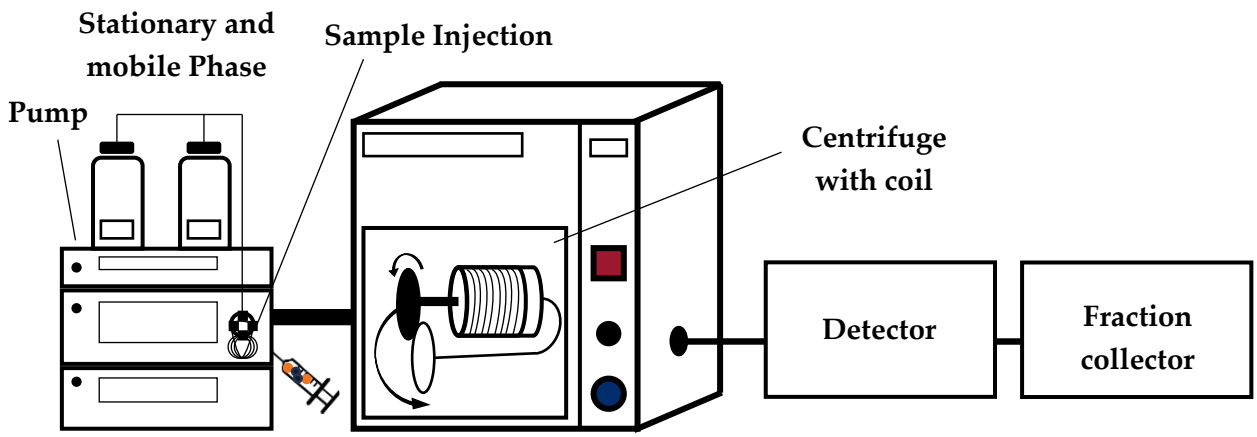


Figure 15: Structural setup of a CCC system. The substances are distributed in the centrifuge and are fractionated and analyzed after the end of the column.

Since there is no solid phase, no irreversible adsorption occurs in this technique. In addition, only low amounts of solvents are necessary, and the resolution is very high [47, 48]. This makes CCC an important method for isolating natural substances from complex mixtures [49, 50].

1.3.2.3. Gas Chromatography

In gas chromatography (GC), the stationary phase is a solid and the mobile phase is gaseous and is also called the carrier gas [39, 40]. The most commonly used capillary columns are made of fused silica with a diameter of ≤ 1 mm. There are several application techniques; however, the sample is always applied on the column as a liquid or a gas. Typical column lengths are 25 m, 50 m or 100 m. The stationary phase is deposited as a thin film on the inner wall of the column. In GC, the column is at the heart of this method and the choice of the column material is crucial for the separation performance. The substances in the column are transported by the inert carrier gas. Typical carrier gases are helium, nitrogen or hydrogen. The target substances

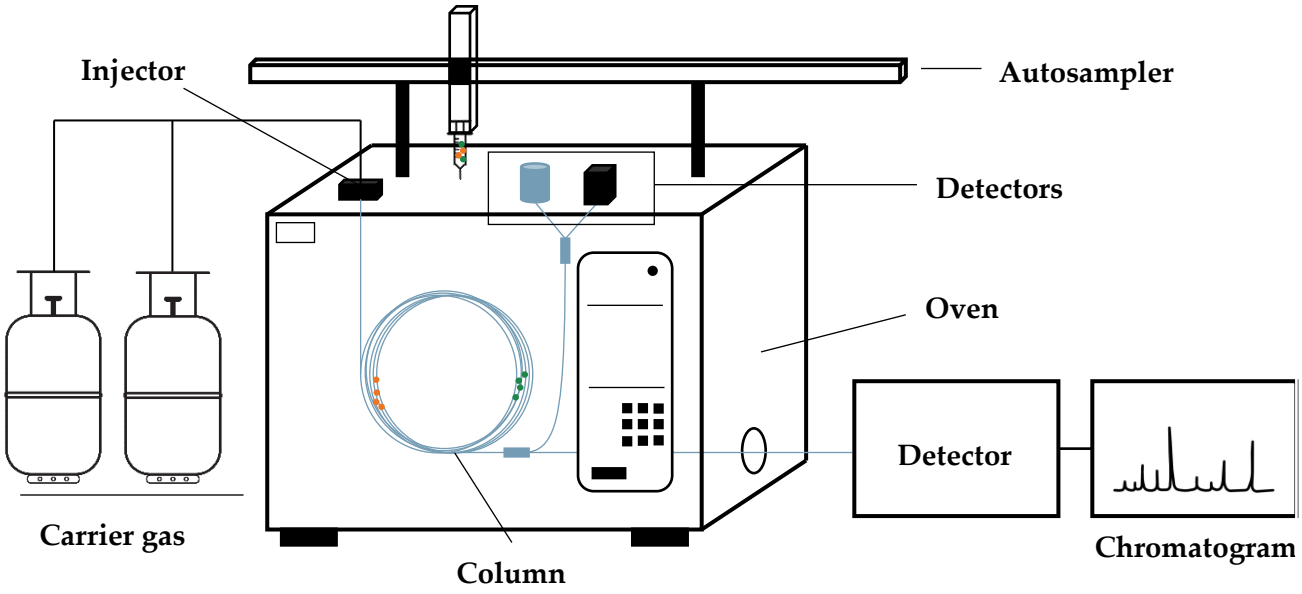


Figure 16: Structural setup of a GC system, equipped with an autosampler and a detector.

are not only separated by substance-specific adsorption processes at the stationary phase but also by the temperature. In the oven, the temperature is substance-specifically tailored to optimize the separation. At the end of the column, the separated substances enter the detector (Figure 16). There are numerous possibilities for detectors in GC, some of them are discussed in the next chapter.

1.3.3. Hyphenated Techniques and Structure Elucidation

Although the application of single chromatographic methods can already lead to the isolation of individual substances, these methods still do not provide any information about the structure of the molecule. Moreover, many substances can only be isolated by repeated or combined separation steps, which applies in particular to naturally occurring substances [40, 41, 51, 52]. The reasons for this are the similarity of a vast number of structures within a compound family. Hence, a single separation step is usually not sufficient. If an interface is required for an online coupling of two instruments to improve the chromatographic separation, this can be distinguished from methods in which only a combination, i.e., a series of methods, is used. Couplings and combinations of chromatographic methods with each other or with molecular spectrometric methods have become an important research tool in the analysis of plants and foods [39, 40, 53-55]. This allows the direct and reproducible combination of isolations and identifications/quantifications. The time-consuming

purification and isolation steps on the laboratory scale can be avoided. Thus, many investigations can be carried out even from small amounts of sample material. Furthermore, by hyphenated techniques higher resolution and throughput can be achieved, which is indispensable for special applications in phytomedicine or in the field of metabolomics.

By definition, a distinction is made between couplings that are not comprehensive, e.g., where only certain fractions are transferred to the second dimension. This is referred to as a "heart-cut" system and is marked with a "-", contrary to a "x", which marks a complete transfer. In view of both approaches, there are various two- and multi-dimensional combination possibilities, such as LC-GC, (GC-)GC-GC or GCxGC, which are primarily used for the isolation of natural products [39, 41].

The coupling or combination of chromatographic methods with molecular spectrometric methods allows not only the isolation of individual substances, but furthermore provides the possibility to conduct identification as well as quantification studies. Nuclear magnetic resonance (NMR), infrared spectroscopy (IR), atomic absorption spectroscopy (AAS) and other spectrometric methods have become fundamental in structure elucidation. There are numerous coupling possibilities for these methods with chromatographic systems, however, UV/Vis and MS are the most established methods for naturally occurring compounds [40, 53, 54].

The principle of UV/Vis spectroscopy is based on the absorption of radiation by molecules [39, 40]. During this process, electrons are excited in the outer orbitals. The wavelength, at which the absorption takes place, is characteristic and allows conclusions about certain structural elements in the molecule. A diode array detector (DAD) is often used for coupling with HPLC. Due to a large number of photodiodes, different wavelengths can simultaneously be detected and thus provide comprehensive spectra.

In MS, ions are separated in the gas phase in a vacuum [39, 40]. By application of high energies (e.g., 70 eV) molecular ions are generated with characteristic mass-to-charge ratios (m/z). Several ionization methods have been established in this respect. For GC analysis, and specifically for the investigation of natural substances, electron ionization (EI) and chemical ionization (CI) are the most frequently used and established methods [39, 53]. In EI, the sample enters the spectrometer via the inlet system and is bombarded with electrons. Radical cations

are formed, and the sample compounds are strongly fragmented. In CI, however, primarily the reaction gas is ionized. This gas reacts subsequently with the sample and the ionization takes place by the transfer of protons or hydride ions [39]. Both EI and CI are carried out under application of vacuum and can therefore be easily coupled with GC. For HPLC, there are various ionization methods that run at atmospheric pressure. Examples are electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI).

After ionization, the sample enters the mass analyzer. Here, the ions are separated according to mass and charge ratio and are then subsequently analyzed. The most commonly used analyzers are quadrupoles, ion traps and time-of-flight (TOF) analyzers. In addition, several quadrupoles can be connected in series. The commonly used triple quadrupoles bring about an increased selectivity and sensitivity. Coupled with chromatographic systems, these techniques can provide extensive characterization of complex samples. GC-MS/MS, GCxGC-TOF-MS or LC-MS/MS are thus often at the heart of the investigation of natural substances [39, 53].

1.4. Metabolization Processes of SQTs in the Human Body

Due to the structural diversity of isoprenoids, a general classification according to nutritional aspects is difficult. However, some of them are essential components of the human nutrition and metabolism, such as vitamin A. On the other hand, potential further positive physiological interactions of SQTs have not yet been investigated in depth. Due to their uptake from external sources such as foods, they can be classified as non-endogenous substances, so-called xenobiotics [2, 56]. Generally, they can be taken up through the skin, the respiratory tract or the mouth [57-59]. The plants investigated in this work are part of the human diet, hence the focus in this chapter is on the metabolization of SQTs during their passage through the gastrointestinal tract (GIT) and their absorption through the intestinal barrier.

1.4.1. Digestion in the GIT

From consumption to excretion, food components pass through the oral cavity, esophagus, stomach, small intestine, large intestine and anus. The GIT also comprises associated systems such as the salivary glands, gastric glands, pancreas, and liver [56, 60]. Besides the mechanical comminution of food and addition of hydrochloric acid, which is produced in the stomach,

enzymes such as amylase, proteases and lipases play the main role in the digestion of food. Thereby, they support the metabolization of the essential food components carbohydrates, proteins, and lipids. Together with vitamins, inorganic salts and water, the metabolization products are mainly absorbed via the small intestine. Two phases of metabolic biotransformation can be distinguished. In phase I, the water solubility of the substances is increased to facilitate excretion [61]. Thereby, the enzyme cytochrome P450 plays an important role in oxidations. Furthermore, there are e.g., epoxidations, reductions, or hydrolysis reactions [57, 61]. In phase II, the substances from phase I are coupled with water-soluble endogenous moieties, such as glutathione, glucuronic acid, and sulphate. All biotransformation products of phases I and II can have different bioactivities than the original substance.

Since digestion studies with human participants are cost- and time-intensive, mainly *in vitro* studies are conducted in this field. Due to the large number of possible individual substances and mixtures to be tested, a significantly higher throughput can be achieved in comparison to *in vivo* studies. In an international consensus, the physiological conditions in the GIT are divided into the three sections oral phase, gastric phase and intestinal phase [62]. In each phase, the typical conditions are mimicked, with the use of enzymes and inorganic salts as well as by adjusting the pH. In a recent study utilizing a simulated digestion system [63], it was shown that some SQTs are metabolized in the human body [23].

By these experiments, the bioaccessibility can be determined, but for the overall picture it is necessary to determine the total bioavailability, which is discussed in the next chapter [59].

1.4.2. Bioavailability and Transport Studies

With a surface area of 200 m^2, the small intestine, which can be divided into the three areas duodenum, jejunum and ileum, is responsible for the absorption of food components [56]. The epithelium of the small intestine consists of the specialized enterocytes, which are interconnected by so-called tight junctions. The enterocytes form microvilli, resulting in the large intestinal surface area. The transport of substances can take place via the transcecullar, paracellular or carrier-mediated pathway or by transcytosis [64, 65].

There are a number of biological and non-biological models to mimic intestinal absorption [65]. An established and standard method is the use of Caco-2 monolayers, which show good correlation with the human jejunum [64-67]. The cell line originates from a human colon epithelial cancer line and forms monolayers upon cultivation, which is usually executed on a semi-permeable membrane (Figure 17). Subsequently, substances are added either on the apical or basolateral side. By determination of the concentration of permeated substances over time, the overall bioavailability can be estimated. The Caco-2 system can be used in drug discovery studies or for xenobiotics in general to determine the permeability, transport and metabolization.

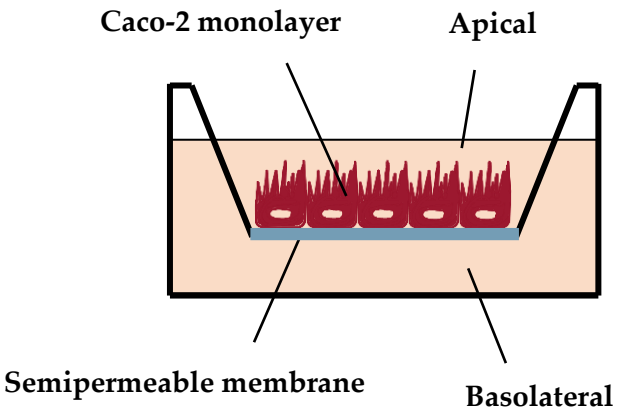


Figure 17: Depiction of a Caco-2 model system. The cells are grown on a semipermeable membrane and the substances can either be added on the apical or basolateral side.

Substances can completely lose or change their physical and chemical properties through metabolization. Knowledge about possible metabolites is thus crucial when investigating the physiological effects of components.

1.5. Physiological Effects of Isoprenoids

1.5.1. Chemosensory Effects

During evolution, humans have developed chemosensors to be able to react to the influences of the outside world [56, 60]. These chemical senses include, amongst others, the senses of smell and taste. The respective chemical information of the substances are detected primarily by G-protein coupled receptors (GPCRs). An exception to this are ion channels, which are responsible for the detection of sour and salty tastes. For sweet, bitter and umami, on the other

hand, there are specific receptor molecules. These receptors, as well as the ion channels, are embedded in the 3000-5000 human taste buds [60]. These structures are embedded in papillae, which are located on the tongue, but also on the palate and in the pharynx.

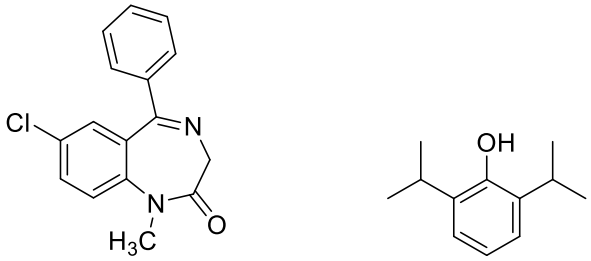
Taste can be clustered into the five sensations salty, sweet, bitter, sour and umami. However, the overall sensation when eating food is referred to as flavor, on which taste and odor have a major influence.

Humans have about 30 million olfactory cells in the olfactory neuroepithelium, localized in the nasal cavity [12, 56, 68, 69]. There are about 350 olfactory receptors (ORs) that are expressed in the cilia of the olfactory sensory neurons. Molecules can bind to several receptors simultaneously, resulting in an individual pattern for an almost infinite number of molecules [70]. These signals are transmitted via the olfactory axons to the olfactory bulb (*bulbus olfactorius*). There, about 1000 signals from olfactory cells of the same specificity are transmitted to the dendrites of mitral cells. This large collection of synapses results in a spherical structure called the glomerulus. Via the axons of the mitral cells (*tractus olfactorius*), the signals are transmitted to various areas in the brain, such as the hippocampus or the orbitofrontal cortex.

From the isoprenoid family, mono- and sesquiterpenes as well as their derivatives are the most odor-active molecules in this substance class. Compounds with more than four isoprene units do not have the volatility, which is a decisive prerequisite for a potentially odor-active substance. A limit of 300 atomic mass units (amu) is the approximate level, above which molecules can no longer be detected by human ORs [70]. Odor-active isoprenoids are of outstanding importance for the aromas of plant products. Due to their high concentration e.g., in EOs, they have a significant influence on their aroma. Some molecules, due to their low odor threshold, can have a predominant influence on a specific aroma [71]. In cases where single substances prime the character of a whole aroma, these are called key aroma compounds. Examples are the monoterpene (R)-limonene in orange juice, the monoterpenoid 1,8-cineol (also called eucalyptol) or the sequiterpenoid muskatone in incense [15, 72]. One focus of current aroma research is the study of structure-odor relationships, where the influence of structure on the odor quality and the odor threshold is investigated [73-77].

1.5.2. $GABA_A$ Receptor Modulation

γ-Aminobutanoic acid (GABA) is the corresponding neurotransmitter of the $GABA_A$ receptor ($GABA_AR$), which is the most important inhibitory neurotransmitter receptor in the central nervous system (CNS) [78]. The receptors are composed of five subunits with 19 different building blocks: α_{1-6}, β_{1-3}, γ, δ, ε, and π. $GABA_ARs$ belong to the family of ligand-gated ion channels. The most important $GABA_AR$ subtype composition is $\alpha_1\beta_2\gamma_2$, and it is estimated that 60 % of all $GABA_ARs$ belong to this subtype. Glycine receptors have a similar structure, but there are fewer building blocks: α_{1-4} and β. $GABA_ARs$ are mainly expressed in the cortex and cerebellum, while glycine receptors are expressed in the brainstem and spinal cord. The five subunits form a central chloride ion-selective channel. The binding of GABA to the receptors leads to an increased influx of chloride ions into the cell. The induced negative charge on the inside of the cell membrane inhibits action potentials, thus inhibits neuronal activity overall [79]. If this mechanism is non functionable, epilepsy, insomnia, anxiety and chronic pain are the consequences [80]. In the protein structure of $GABA_ARs$, there are binding sites, where allosteric modulators can interact and change the configuration of the receptor. On the one hand, there are modulators with a negative effect on the GABAergic currents. For example, with the co-application of picrotoxin and GABA, fewer chloride ions can penetrate the cell compared to the pure application of GABA. The incoming action potentials can thus no longer be sufficiently inhibited. On the other hand, there are substances, which co-application leads to an increased influx of chloride ions into the cell. Due to the importance of $GABA_ARs$ for the CNS, there has been a lot of research to identify allosteric modulators and their enormous pharmacological significance. Well-established positive modulators are e.g., the substance classes of benzodiazepines, barbiturates and endogenous neurosteroids [78, 79]. Diazepam, which is a member of the benzodiazepine family is one of the most used pharmaceutical substance, however the disadvantages can be psychological dependence in the case of long-term use [78]. Propofol is another example for a commonly used general anesthetic [81] (Figure 18).



Diazepam Propofol

Figure 18: Structures of Diazepam (left) and Propofol (right), which are two well-known GABA$_A$R modulators.

Many mechanisms of allosteric modulators are not yet understood. In recent years, several crystal structures of GABA$_A$Rs have been elucidated, thus enabling docking studies [79, 80, 82, 83]. However, there are also many modulators among naturally occurring substances. The possible synergistic effects that can arise from the interaction at several binding sites of different substances are as well not yet fully understood.

Modulatory effects on GABA$_A$Rs by secondary plant metabolites have been shown in previous studies, e.g., for naturally occurring substances [84-86], for terpenes and terpenoids [87-90] and also for SQTs [88, 91]. With tailored modification and the alteration of specific moieties of the molecule, even higher modulation can be achieved. This was shown for valerenic acid, where the addition of nitrogen-containing moieties was accompanied by an increase of the GABAergic current [91].

As described in chapter 1.4, metabolization can comprise derivatization with oxygen containing moieties in phase I. The hydrophilicity or lipophilicity of substances is crucial for their absorption and further distribution in the human body, including the transport through the blood-brain barrier. For GABA$_A$R studies, it is therefore of interest also to test the metabolites, as these may have an altered modulatory potential. This change of the modulatory potential was recently investigated for linaool and some derivatives [92]. While linalool enhanced the GABAergic currents, the coapplication of GABA with 8-hydroxylinalool or 8-hydroxylinalyl acetate respectively, led to a decrease of the currents. Other investigated compounds like 8-oxolinalool share the same positive potential as linaool.

1.6. Perspectives: Potentials for Future Applications

EOs, and thus isoprenoids in particular, have become indispensable in many contemporary applications. Soft drinks, fragrances, household chemicals, cosmetics or pharmaceuticals are just a few of the many areas of application [57]. On the one hand, EOs are used in many products primarily because of their smell. On the other hand, the diverse and positive physiological and biological effects are used in many applications: for example, antibacterial properties which are used to increase the shelf life of foodstuffs [93].

The anti-inflammatory, antimicrobial and antioxidant properties of EOs in particular lead to a potentially huge number of applications [6, 93]. A future-oriented production is supported by the advantage of EOs being natural substances in general. In combination with suitable biomaterials, applications fulfil the requirements of a carbon-neutral, renewable, and sustainable circular economy [94]. Due to the many positive physiological effects of EOs, they can be combined with biomedical applications in humans. Biomaterials in general are used for example in controlled drug delivery systems [95]. Biodegradability and biocompatibility, as well as the general high adaptivity to the biological environment are nowadays the focus for applications in the field of tissue engineering [95, 96]. One example is the use of essential oils in bone-healing applications [96, 97]. Previous approaches have often led to problems due to microbial contamination. By combining biomaterials with EOs, these problems might be avoided. Furthermore, this could provide applications for diseases such as osteoporosis and osteoarthritis. Another example is the use of EOs in wound-healing applications [98-101]. On the one side, the healing process can potentially be accelerated, but also solutions could be found for chronic wounds such as diabetic foot ulcers. The challenge, when incorporating EOs, is to find a tailored material that optimally supports the healing process with a high adaptivity to the environment. In addition, the material must release the EOs in a controlled manner and in sufficient concentrations over a suitable period of time. For this, it is crucial to find comprehensive analytical solutions to monitor this release and thus to contribute to a thorough knowledge on these materials.

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2. Aims and Outline

The exceptionality of nature is reflected, among other things, in the almost infinite number of secondary plant metabolites, which serve as communication tools with the environment. One of the most important and frequently occurring substance classes are isoprenoids, which can be characterized by the common building block of the isoprene unit. Biosynthesis from these building blocks almost exclusively results in complex structures, making chemical syntheses, compound isolations and characterizations a challenging task. Within the substance class of isoprenoids, SQTs, consisting of three isoprene units, are often the main representatives characterizing EOs, and these thus represent an important subgroup thereof. Plants, especially those containing EOs, have always been important pharmaceutical ingredients of humankind. First, they were used in traditional medicine and are nowadays, due to the technical progress and evidence-based approaches, also utilized in modern medicine. It is therefore of great importance to gain deeper knowledge about these molecules, and their physiological effects, but also to develop strategies that allow their isolation directly from promising natural sources.

To achieve this goal, the aim of the current study was to, firstly, identify and select representative plants that are promising targets in this respect, and should be comprehensively characterized specifically with respect to their SQTs. Since only a few SQTs in general are available synthetically or commercially, the first steps of this work should thus cover identifications and isolations of these substances. Thereby, the focus should not be placed on single isolation techniques, but rather on using combined and hyphenated approaches and methods to achieve a targeted and rapid isolation. One aim was to preferentially explore chromatographic techniques, like CCC, which has already proven its strengths in the targeted isolation of natural substances in the past. The substances isolated in this way, together with other commercially available SQTs, should then be examined according to their metabolization and absorption in the human body. With this knowledge of bioavailability, the compounds should then be investigated in a next step to study the influence on the human $GABA_AR$. Different subtypes of the receptor, as well as different combinations of target substances will be used to build up a profound understanding of the neurotropic effects of SQTs. The overall results will provide a comprehensive picture of the

physiological interaction of SQTs and serve as a basis for a sustainable, evidence-based phytomedicine.

In **chapter 3.1**, SQTs of five selected plants should be identified by means of GC-MS. Comparison of the mass spectra with databases should utilize unambiguous assignment of detector signals. Furthermore, the SQTs should be quantified by using several target-oriented internal standards. In c**hapter 3.2** suitable plant materials and extracts should be taken for isolation of promising substances with regard to modulating GABAergic currents. By using combinations of chromatographic techniques, with the focus on countercurrent chromatography, isolations with high yields and purity should be achieved. The compounds isolated from chapter 3.2. together with commercially and synthetically availably compounds should then be investigated in **chapter 3.3** for their $GABA_AR$ modulatory potential. By focusing on several $GABA_AR$ subtypes a comprehensive understanding of the modulatory potential in the human CNR should be reached. The analytical knowledge for SQTs from the previous chapters should then be further tailored to characterize different biomaterials. These materials should be made of Poly(ε-caprolactone)/gelatin or bioactive glass/soy protein spiked with clove essential oil (**chapter 3.4**), peppermint essential oil (**chapter 3.5**) and cinnamon essential oil (**chapter 3.6**).

3. List of Publications and Author Contributions

3.1. Publication 1

Slavik, B.; Nehr, J.; Loos, H. M.; Buettner, A.; Identification and Semi-quantification of Sesquiterpenes and Sesquiterpenoids from Chamomile, Hop, Lavender, Basil and Lemon Balm. Proceedings of the 16th Weurman Flavour Research Symposium (2021), doi: 10.5281/zenodo.5752298

B. Slavik set up the experimental design. J. Nehr performed the experiments and analysed the data. B. Slavik, J. Nehr, H. M. Loos and A. Buettner evaluated and interpreted the data. B. Slavik wrote the manuscript. J. Nehr, H. M. Loos and A. Buettner revised and edited the manuscript.

3.2. Publication 2

Slavik, B.; Roehrer, S.; Loos, H. M.; Minceva, M.; Buettner, A., Isolation of sesquiterpenoids from Matricaria chamomilla by means of solvent assisted flavor evaporation and centrifugal partition chromatography. Anal Bioanal Chem 2021, 413 (17), 4387-4396, doi: 10.1007/s00216-021-03400-w, IF 3.637.

B. Slavik performed the extraction, SAFE, purification and analytical experiments. S. Roehrer was responsible for the simulation experiments and together with B. Slavik performed the CCC and CPC separation experiments. B. Slavik evaluated and interpreted the data together with H. M. Loos and S. Roehrer. B. Slavik, S. Roehrer, H. M. Loos, M. Minceva and A. Buettner contributed to the manuscript and conceived and planned the study.

3.3. Publication 3

Janzen, D.; **Slavik, B.**; Zehe, M.; Sotriffer, C.; Loos, H. M.; Buettner, A.; Villmann, C., Sesquiterpenes and sesquiterpenoids harbor modulatory allosteric potential and affect inhibitory GABAA receptor function in vitro. J Neurochem 2021, 159 (1), 101-115, doi: 10.1111/jnc.15469, IF 5.372.

C. Villmann and A. Büttner participated in research design. Electrophysiology was performed by D. Janzen. GC-MS and isolation of SQTs was performed by B. Slavik. D. Janzen, C. Villmann, B. Slavik, and H. Loos carried out data analysis. M. Zehe and C. Sotriffer carried out modeling and docking analysis. C. Villmann, D. Janzen, and B. Slavik contributed to the writing of the manuscript.

3.4. Publication 4

Unalan, I.; Endlein, S. J.; **Slavik, B.**; Buettner, A.; Goldmann, W. H.; Detsch, R.; Boccaccini, A. R., Evaluation of Electrospun Poly(ε-Caprolactone)/Gelatin Nanofiber Mats Containing Clove Essential Oil for Antibacterial Wound Dressing. Pharmaceutics 2019, 11 (11), doi: 10.3390/pharmaceutics11110570, IF 4.773.

Irem Unalan conceived the original idea and carried out the conceptualization, methodology, validation, literature survey, formal analysis, investigation, data curation, visualization, and writing – of the initial draft. Stefan J Endlein participated in formal analysis and investigation. Benedikt Slavik conceived and measured the eugenol calibration curve. Additionally, Benedikt Slavik designed the SAFE procedure, Vigreux- and micro-distillation, and GC-MS analysis process as described in section 2.3 in the manuscript. The first author performed the sample preparation, the SAFE procedure, and Vigreux- and micro-distillation and data analysis. Andrea Buettner carried out resources, supervision, and funding acquisition for GC-MS analysis. Wolfgang H Goldmann helped with the supervision of antibacterial analysis. Rainer Detsch helped with the supervision of cell studies. Aldo R. Boccaccini carried out supervision, project administration, and funding acquisition. All authors read and approved the final manuscript.

3.5. Publication 5

Unalan, I.; **Slavik, B.**; Buettner, A.; Goldmann, W. H.; Frank, G.; Boccaccini, A. R., Physical and Antibacterial Properties of Peppermint Essential Oil Loaded Poly (epsilon-caprolactone) (PCL) Electrospun Fiber Mats for Wound Healing. Front Bioeng Biotechnol 2019, 7, 346, doi: 10.3389/fbioe.2019.00346, IF 5.122.

Irem Unalan conceived the original idea and carried out the conceptualization, methodology, validation, literature survey, formal analysis, investigation, data curation, visualization, and writing – of the initial draft. Benedikt Slavik conceived and carried out the identification of the volatile compound of peppermint oil (PEP) by GC-MS analysis and measurement of the menthol calibration curve. Additionally, Benedikt Slavik conceived the SAFE procedure, Vigreux- and micro-distillation, and GC-MS analysis process as described in section "Peppermint oil content in PCL electrospun fiber mats" in the manuscript. The first author performed the sample preparation, the SAFE procedure, and Vigreux- and micro-distillation and data analysis. Andrea Buettner carried out resources, supervision, and funding acquisition for GC-MS analysis. Wolfgang H Goldmann helped with the supervision of antibacterial analysis. Gerhard Frank helped with the supervision of Raman spectroscopy analysis and carried out pure peppermint oil analysis. Aldo R. Boccaccini carried out supervision, project administration, and funding acquisition. All authors read and approved the final manuscript.

3.6. Publication 6

Unalan, I.; Fuggerer, T.; **Slavik, B.**; Buettner, A.; Boccaccini, A. R., Antibacterial and antioxidant activity of cinnamon essential oil-laden 45S5 bioactive glass/soy protein composite scaffolds for the treatment of bone infections and oxidative stress. Mater Sci Eng C Mater Biol Appl 2021, 128, 112320, doi: 10.1016/j.msec.2021.112320, IF: 7.328.

Irem Unalan conceived the original idea and carried out the conceptualization, methodology, validation, literature survey, formal analysis, investigation, data curation, visualization, and writing – of the initial draft. Tim Fuggerer participated formal analysis and investigation. Benedikt Slavik conceived and measured the cinnamaldehyde calibration curve. Additionally, Benedikt Slavik designed the SAFE procedure, Vigreux- and micro-distillation, and GC-MS analysis process as described in section 2.4 in the manuscript. The first author performed the sample preparation, the SAFE procedure, and Vigreux- and micro-distillation and data analysis. Andrea Buettner carried out resources, supervision, and funding acquisition for GC-MS analysis. Aldo R. Boccaccini carried out supervision, project administration, and funding acquisition. All authors read and approved the final manuscript.

3.7. Further Publications (not peer-reviewed)

B. Slavik, S. Roehrer, H.M. Loos, M. Minceva, A. Buettner. Isolierung von Sesquiterpenoiden aus Matricaria chamomilla durch Kombination chromatographischer Methoden. 71. Arbeitstagung des Regionalverbandes Bayern der Lebensmittelchemischen Gesellschaft, Würzburg, 10.03.2020.

B. Slavik, D. Janzen, S. Roehrer, S. V. Luca, T. Stroebel, C. Fey, H. M. Loos, M. Minceva, C. Villmann, A. Buettner. Sesquiterpene und Sesquiterpenoide in Kräutern und Blüten – Untersuchungen zu ihrem Vorkommen und ihrer physiologischen Wirkung. 49. Deutscher Lebensmittelchemikertag, 30.08. – 01.09.2021.

B. Slavik, S. Roehrer, J. Nehr, H.M. Loos, M. Minceva, A. Buettner. Sesquiterpenes and sesquiterpenoids: Exploring combinatorial approaches of chromatographic techniques for their targeted isolation. 16th Weurman Flavour Research Symposium, 04.-06.05.2021.

4. Conclusion and Outlook

The large number of naturally occurring compounds and their complex structure are the major challenges in identifications and isolations. Even when focusing on single compound classes, like SQTs, the challenges remain. Nevertheless, in this investigation, five plants were selected and more than 50 different SQTs could be identified and partially quantified. The plants investigated were hop (*Humulus lupulus* L.), lavender (*Lavandula angustifolia*), lemon balm *(Melissa officinalis* L.), chamomile (*Matricaria chamomilla* L.) and basil (*Ocimum basilicum* L.). A suitable method is GC-MS, which can also be used to chromatographically separate very similar sesquiterpenes. The challenge, however, lies in the unambiguous identification since many SQTs, which all have similar chemical structures, have an analogical mass spectrum. In addition to one-dimensional and two-dimensional GC coupled with mass spectrometry, a large number of already elucidated structures could be identified via suitable reference databases. Due to the improvement of mass spectrometers, e.g., TOF-MS, the level of identification and the level of quantification is shifting to lower thresholds. On the other hand, other techniques are needed to isolate larger quantities that could be applied in various (bio-)chemical assays. In this work, a number of sesquiterpenoids were successfully isolated with the CCC/CPC technique, where a large throughput could be achieved. The technique has been shown to be a promising tool in such challenging separation tasks. However, in the future, the methods must be further adapted regarding to individual components. In this respect, modern chromatographic systems and a targeted isolation strategy might lead to more readily available SQTs in the future. For example, comprehensive couplings of LC, CCC and GC might serve as suitable isolation tools.

Even if numerous natural substances have not yet been identified or isolated so far, general knowledge about the compound structures is already existing for many plants. However, there is little knowledge about metabolism and uptake processes of natural occurring substances in the human body. Among other things, the acidic environment in the stomach might lead to metabolic changes in the absorbed molecules and greatly alter their biochemical properties. The metabolism and bioavailability are crucial parameters when it comes to the investigation of the physiological and receptor interactions of the molecules. The plants investigated in this project are known in traditional medicine and in current investigations for

their calming, sleep-inducing and sedative properties. These effects are mediated by the human $GABA_AR$ the most important inhibitory receptor in the CNS. Different components, including binary mixtures, have been studied on different receptor types. Both, positive and negative modulators of chloride ion influx were found. It was shown that the γ-subtype plays an important role by the modulation of SQTs.

The knowledge gained from this work regarding the identification and quantification of natural occurring compounds was used as basis to characterize various biomaterials. By different approaches quantifications of peppermint EO, clove EO and cinnamon EO were achieved. The aim of the EO incorporated biomaterials is to take advantage of the positive bioactive properties and thus accelerate wound and bone healing.

Nature's secrets have been partially deciphered, but many ambiguities remain. For the analytical chemistry in particular, many challenges in the proper characterization of plant compounds are still to overcome. New techniques need to be tested and validated and combined with existing methods to provide a comprehensive understanding and thus being able to contribute to unraveling the still manifold secrets of nature.