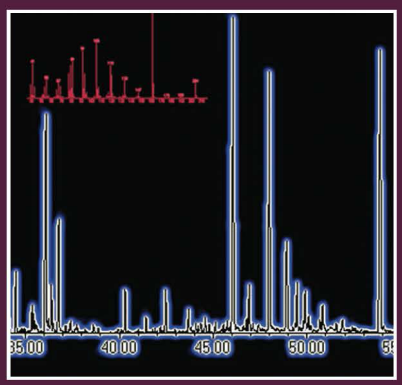
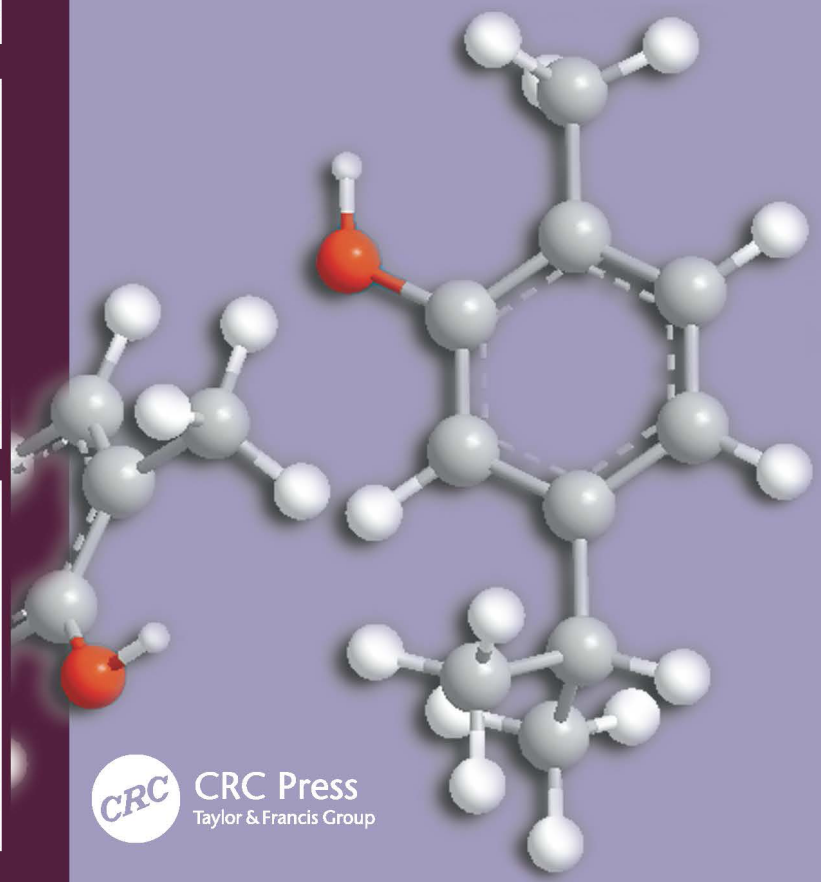
Handbook of ESSENTIAL OILS

Science, Technology, and Applications

THIRD EDITION

Edited by
K. Hüsnü Can Başer
Gerhard Buchbauer





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Editors

K. Hüsnü Can Başer was born on July 15, 1949, in Çankırı, Turkey. He graduated from the Eskisehir I.T.I.A. School of Pharmacy with diploma number 1 in 1972 and became a research assistant in the Pharmacognosy Department of the same school. He did his PhD in pharmacognosy between 1974 and 1978 at Chelsea College of the University of London. Upon returning home, he worked as a lecturer in pharmacognosy at the school from which he had earlier graduated and served as director of Eskisehir I.T.I.A. School of Chemical Engineering between 1978 and 1980. He was promoted to associate professorship in pharmacognosy in 1981. He served as dean of the Faculty of Pharmacy at Anadolu University (1993–2001), vice-dean of the Faculty of Pharmacy (1982–1993), head of the Department of Professional Pharmaceutical Sciences (1982–1993), head of the Pharmacognosy Department (1982–2011), member of the University Board and Senate (1982–2001; 2007), and director of the Medicinal and Aromatic Plant and Drug Research Centre (TBAM) (1980–2002) in Anadolu University.

During 1984–1994, he was appointed as the national project coordinator of Phase I and Phase II of the UNDP/UNIDO projects of the government of Turkey titled "Production of Pharmaceutical Materials from Medicinal and Aromatic Plants," through which TBAM had been strengthened. He was promoted to full professorship in pharmacognosy in 1987. After his early retirement from Anadolu University in 2011, he served as visiting professor in King Saud University in Riyadh, Saudi Arabia (2011–2015). He is currently working as head of the Pharmacognosy Department in the Faculty of Pharmacy of Near East University in Nicosia, N. Cyprus, and director of the Graduate Institute of Health Sciences of the same university. His major areas of research include essential oils, alkaloids, and biological, chemical, pharmacological, technological, and biological activity research into natural products. He is the 1995 Recipient of the Distinguished Service Medal of IFEAT (International Federation of Essential Oils and Aroma Trades) based in London, United Kingdom, and the 2005 recipient of "Science Award" (Health Sciences) of the Scientific and Technological Research Council of Turkey (TUBITAK), which are among the 18 awards he has been bestowed so far. He is listed among the 100 Turks Leading Science. He has published 827 research papers in international refereed journals, 184 papers in Turkish journals, and 141 papers in conference proceedings. He has published altogether 1212 scientific contributions as papers, books, or book chapters. According to SCI, his 594 papers were cited 10,453 times. His H-index is 47. According to Google Scholar, his publications were cited 26,540 times. His H-index is 70; i10 index is 552. His 5 books were published in Turkey, Japan, the UK, and the US (2).

More information can be found at http://www.khcbaser.com.

Gerhard Buchbauer was born in 1943 in Vienna, Austria. He studied pharmacy at the University of Vienna, from where he received his master's degree (Mag. pharm.) in May 1966. In September 1966, he assumed the duties of university assistant at the Institute of Pharmaceutical Chemistry and received his doctorate (PhD) in pharmacy and philosophy in October 1971, with a thesis on synthetic fragrance compounds. Further scientific education was practiced postdoc in the team of Professor C. H. Eugster at the Institute of Organic Chemistry, University of Zurich (1977–1978), followed by the habilitation (postdoctoral lecture qualification) in pharmaceutical chemistry with the inaugural dissertation entitled "Synthesis of Analogies of Drugs and Fragrance Compounds with Contributions to Structure-Activity-Relationships" (1979) and appointment as permanent staff of the University of Vienna and head of the first department of the Institute of Pharmaceutical Chemistry.

In November 1991, he was appointed as a full professor of pharmaceutical chemistry, University of Vienna; in 2002, he was elected as head of this institute. He retired in October 2008. He has been married since 1973 and had a son in 1974.

Among others, he is still a member of the permanent scientific committee of International Symposium on Essential Oils (ISEO); a member of the scientific committee of Forum Cosmeticum (1990, 1996, 2002, and 2008); a member of numerous editorial boards (e.g., *Journal of Essential Oil Research*, the *International Journal of Essential Oil Therapeutics*, *Scientia Pharmaceutica*); assistant editor of *Flavour and Fragrance Journal*; regional editor of *Eurocosmetics*; a member of many scientific societies (e.g., Society of Austrian Chemists, head of its working group "Food Chemistry, Cosmetics, and Tensides" [2000–2004]; Austrian Pharmaceutical Society; Austrian Phytochemical Society; vice head of the Austrian Society of Scientific Aromatherapy; and so on); technical advisor of IFEAT (1992–2008); and organizer of the 27th ISEO (September 2006, in Vienna) together with Professor Dr. Ch. Franz and senior advisor at the 50th ISEO (September 2019), organized in Vienna again.

Based on the sound interdisciplinary education of pharmacists, it was possible to establish an almost completely neglected area of fragrance and avor chemistry as a new research discipline within the pharmaceutical sciences. Our research team is the only one that conducts fragrance research in its entirety and covers synthesis, computer-aided fragrance design, analysis, and pharmaceutical/medicinal aspects. Because of our efforts, it is possible to show and to prove that these small molecules possess more properties than merely emitting a good odor. Now, this research team has gained a worldwide scientific reputation documented by more than 450 scientific publications, about 100 invited lectures, and about 200 contributions to symposia, meetings, and congresses, as short lectures and poster presentations.

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1 Introduction

K. Hüsnü Can Başer and Gerhard Buchbauer

The overwhelming success of the first edition of the *Handbook of Essential Oils: Science, Technology, and Applications* had urged the publication of the second edition which was bestowed, in 2016, the *ABC James A. Duke Excellence in Botanical Literature Award* for the excellent contribution to the vast field of essential oils. This prestigious award by the *American Botanical Council* for the best book in botanical literature has prompted us to prepare a third edition of this Handbook.

As in the previous edition, updated chapters as well as completely new chapters have been included in the third edition. Some important chapters remained as such. Thus, we kept the contributions of the current Chapters 2, 5, 6, 9, 22, 23, 25, 32, and 33 as in the second edition. We skipped Chapters 15, 16, and 26 in the second edition of the *Handbook*, whereby the former Part Chapter 16, "Aromatherapy with Essential Oils", has been substituted by Rhiannon Lewis (Chapter 13). In this edition, Chapters 4, 7, 10, 26, 30, and 31 have been updated, and many new contributions have been added, covering the commonly entitled "Biological activities of…" chapters in the form of six chapters. These are "Essential Oils in Cancer Therapy" (Chapter 14), then "Antimicrobial Activity of Selected Essential Oils and Aromas" (Chapter 15), followed by "Quorum Sensing and Essential Oils" (Chapter 16), then "Essential Oils as Carrier Oils" (Chapter 27), and then two new (more chemically written) overviews, namely "Influence of Light on Essential Oil Constituents" and "Influence of Air on Essential Oil Constituents" (now Chapters 28 and 29). The new Chapter 19, entitled "Adverse Effects and Intoxication with Essential Oils" is an overview written by a pharmacologist of the University of Vienna. The former Chapter 12 now has been substituted by the updated chapter "Central Nervous System Effects of Essential Oil Compounds" (now Chapter 11) and another, newly entitled, treatise, namely "Effects of Essential Oils on Human Cognition" (now Chapter 12). "Essential Oils and Volatiles in Bryophytes" is a new chapter (Chapter 21) by Agnieszka Ludwiczuk and Yoshinori Asakawa. "Functions of Essential Oils and Natural Volatiles in Plant–Insect Interactions" (Chapter 17) was contributed by R. Raguso. "Essential Oils as Lures for Invasive Ambrosia Beetles" (Chapter 18) is yet another new contribution. A useful new chapter for GC/MS analysts is entitled "Use of Linear Retention Indices in GC/MS Libraries for Essential Oil Analysis" (Chapter 8).

Also with this third edition, we hope that many scientists, especially in the fields of essential oils in botany, chemistry, pharmacognosy, medicine, clinical aromatherapy, and other relevant aspects of these natural products, will find these contributions not only alluring for their own research but also interesting to read and to find out what manifold properties essential oils have. Especially, also in this third edition, we want to provide a strong scientific basis for essential oils and to prevent any trace of esoteric ignorance.

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2 History and Sources of Essential Oil Research

Karl-Heinz Kubeczka

CONTENTS

2.1 ANCIENT HISTORICAL BACKGROUND

Plants containing essential oils have been used since furthest antiquities as spices and remedies for the treatment of diseases and in religious ceremonies because of their healing properties and their pleasant odors. In spite of the obscured beginning of the use of aromatic plants in prehistoric times to prevent, palliate, or heal sicknesses, pollen analyses of Stone Age settlements indicate the use of aromatic plants that may be dated to 10,000 BC.

One of the most important medical documents of ancient Egypt is the so-called Papyrus Ebers of about 1550 BC, a 20 m long papyrus, which was purchased in 1872 by the German Egyptologist

G. Ebers, for whom it is named, containing some 700 formulas and remedies, including aromatic plants and plant products like anise, fennel, coriander, thyme, frankincense, and myrrh. Much later, the ancient Greek physician Hippocrates (460–377 BC), who is referred to as the father of medicine, mentioned in his treatise *Corpus Hippocratium* approximately 200 medicinal plants inclusive of aromatic plants and described their efficacies.

One of the most important herbal books in history is the five-volume book *De Materia Medica*, written by the Greek physician and botanist Pedanius Dioscorides (ca. 40–90), who practiced in ancient Rome. In the course of his numerous travels all over the Roman and Greek world seeking for medicinal plants, he described more than 500 medicinal plants and respective remedies. His treatise, which may be considered a precursor of modern pharmacopoeias, was later translated into a variety of languages. Dioscorides, as well as his contemporary Pliny the Elder (23–79), a Roman natural historian, mention besides other facts turpentine oil and give some limited information on the methods in its preparation.

Many new medicines and ointments were brought from the east during the Crusades from the eleventh to the thirteenth centuries, and many herbals, whose contents included recipes for the use and manufacture of essential oil, were written during the fourteenth to the sixteenth centuries.

Theophrastus von Hohenheim, known under the name Paracelsus (1493–1541), a physician and alchemist of the fifteenth century, defined the role of alchemy by developing medicines and extracts from healing plants. He believed distillation released the most desirable part of the plant, the *Quinta essentia* or *quintessence* by a means of separating the "essential" part from the "nonessential" containing its subtle and essential constituents. The currently used term "essential oil" still refers to the theory of *Quinta essentia* of Paracelsus.

The roots of distillation methods are attributed to Arabian Alchemists centuries with Avicenna (980–1037) describing the process of steam distillation, who is credited with inventing a coiled cooling pipe to prepare essential oils and aromatic waters. The first description of distilling essential oils is generally attributed to the Spanish physician Arnaldus de Villa Nova (1235–1311) in the thirteenth century. However, in 1975, a perfectly preserved terracotta apparatus was found in the Indus Valley, which is dated to about 3000 BC and which is now displayed in a museum in Taxila, Pakistan. It looks like a primitive still and was presumable used to prepare aromatic waters. Further findings indicate that distillation has also been practiced in ancient Turkey, Persia, and India as far back as 3000 BC.

At the beginning of the sixteenth century appeared a comprehensive treatise on distillation by Hieronymus Brunschwig (ca. 1450–1512), a physician of Strasbourg. He described the process of distillation and the different types of stills in his book *Liber de arte Distillandi de compositis* (Strasbourg 1500 and 1507) with numerous block prints. Although obviously endeavoring to cover the entire field of distillation techniques, he mentions in his book only the four essential oils from rosemary, spike lavender, juniper wood, and the turpentine oil. Just before, until the Middle Ages, the art of distillation was used mainly for the preparation of aromatic waters, and the essential oil appearing on the surface of the distilled water was regarded as an undesirable by-product.

In 1551 appeared at Frankfurt on the Main the *Kräuterbuch*, written by Adam Lonicer (1528–1586), which can be regarded as a significant turning point in the understanding of the nature and the importance of essential oils. He stresses that the art of distillation is a quite recent invention and not an ancient invention and has not been used earlier.

In the *Dispensatorium Pharmacopolarum* of Valerius Cordus, published in Nuremberg in 1546, only three essential were listed; however, the second official edition of the *Dispensatorium Valerii Cordi* issued in 1592, 61 distilled oils were listed illustrating the rapid development and acceptance of essential oils. In that time, the so-called Florentine flask has already been used for separating the essential oil from the water phase.

The German J.R. Glauber (1604–1670), who can be regarded as one of the first great industrial chemists, was born in the little town Karlstadt close to Wuerzburg. His improvements in chemistry, for example, the production of sodium sulfate, as a safe laxative brought him the honor of being named Glauber's salt. In addition, he improved numerous different other chemical processes and especially new distillation devices also for the preparation of essential oils from aromatic plants. However, it lasted until the nineteenth century to get any real understanding of the composition of true essential oils.

2.2 FIRST SYSTEMATIC INVESTIGATIONS

The first systematic investigations of constituents from essential oils may be attributed to the French chemist M.J. Dumas (1800–1884) who analyzed some hydrocarbons and oxygen as well as sulfur- and nitrogen-containing constituents. He published his results in 1833. The French researcher M. Berthelot (1859) characterized several natural substances and their rearrangement products by optical rotation. However, the most important investigations have been performed by O. Wallach, an assistant of Kekule. He realized that several terpenes described under different names according to their botanical sources were often, in fact, chemically identical. He, therefore, tried to isolate the individual oil constituents and to study their basic properties. He employed together with his highly qualified coworkers Hesse, Gildemeister, Betram, Walbaum, Wienhaus, and others fractional distillation to separate essential oils and performed reactions with inorganic reagents to characterize the obtained individual fractions. The reagents he used were hydrochloric acid, oxides of nitrogen, bromine, and nitrosyl chloride—which was used for the first time by W.A. Tilden (1875)—by which frequently crystalline products had been obtained.

At that time, hydrocarbons occurring in essential oils with the molecular formula $C_{10}H_{16}$ were known, which had been named by Kekule *terpenes* because of their occurrence in turpentine oil. Constituents with the molecular formulas $C_{10}H_{16}O$ and $C_{10}H_{18}O$ were also known at that time under the generic name camphor and were obviously related to terpenes. The prototype of this group was camphor itself, which was known since antiquity. In 1891, Wallach characterized the terpenes pinene, camphene, limonene, dipentene, phellandrene, terpinolene, fenchene, and sylvestrene, which has later been recognized to be an artifact.

During 1884–1914, Wallach wrote about 180 articles that are summarized in his book *Terpene und Campher* (Wallach, 1914) compiling all the knowledge on terpenes at that time, and already in 1887, he suggested that the terpenes must be constructed from isoprene units. In 1910, he was honored with the Nobel Prize for Chemistry "in recognition of his outstanding research in organic chemistry and especially in the field of alicyclic compounds" (Laylin, 1993).

In addition to Wallach, the German chemist A. von Baeyer, who also had been trained in Kekule's laboratory, was one of the first chemists to become convinced of the achievements of structural chemistry and who developed and applied it to all of his work covering a broad scope of organic chemistry. Since 1893, he devoted considerable work to the structures and properties of cyclic terpenes (von Baeyer and Seuffert, 1901). Besides his contributions to several dyes, the investigations of polyacetylenes, and so on, his contributions to theoretical chemistry including the strain theory of triple bonds and small carbon cycles have to be mentioned. In 1905, he was awarded the Nobel Prize for Chemistry "in recognition of his contributions to the development of Organic Chemistry and Industrial Chemistry, by his work on organic dyes and hydroaromatic compounds" (Laylin, 1993). The frequently occurring acyclic monoterpenes geraniol, linalool, citral, and so on have been investigated by F.W. Semmler and the Russian chemist G. Wagner (1899), who recognized the importance of rearrangements for the elucidation of chemical constitution, especially the carbon-to-carbon migration of alkyl, aryl, or hydride ions, a type of reaction that was later generalized by H. Meerwein (1914) as Wagner–Meerwein rearrangement.

More recent investigations of J. Read, W. Hückel, H. Schmidt, W. Treibs, and V. Prelog were mainly devoted to disentangle the stereochemical structures of menthols, carvomenthols, borneols, fenchols, and pinocampheols, as well as the related ketones (see Gildemeister and Hoffmann, 1956).

A significant improvement in structure elucidation was the application of dehydrogenation of sesqui- and diterpenes with sulfur and later with selenium to give aromatic compounds as a major method, and the application of the isoprene rule to terpene chemistry, which have been very efficiently used by L. Ruzicka (1953) in Zurich, Switzerland. In 1939, he was honored in recognition of his outstanding investigations with the Nobel Prize in chemistry for his work on "polymethylenes and higher terpenes."

The structure of the frequently occurring bicyclic sesquiterpene ß-caryophyllene was for many years a matter of doubt. After numerous investigations, W. Treibs (1952) has been able to isolate the crystalline caryophyllene epoxide from the autoxidation products of clove oil, and F. Šorm et al.

(1950) suggested caryophyllene to have a four- and nine-membered ring on bases of infrared (IR) investigations. This suggestion was later confirmed by the English chemist D.H.R. Barton (Barton and Lindsay, 1951), who was awarded the Nobel Prize in Chemistry in 1969.

The application of ultraviolet (UV) spectroscopy in the elucidation of the structure of terpenes and other natural products was extensively used by R.B. Woodward in the early forties of the last century. On the basis of his large collection of empirical data, he developed a series of rules (later called the Woodward rules), which could be applied to finding out the structures of new natural substances by correlations between the position of UV maximum absorption and the substitution pattern of a diene or an α,β-unsaturated ketone (Woodward, 1941). He was awarded the Nobel Prize in Chemistry in 1965. However, it was not until the introduction of chromatographic separation methods and nuclear magnetic resonance (NMR) spectroscopy into organic chemistry that a lot of further structures of terpenes were elucidated. The almost exponential growth in our knowledge in that field and other essential oil constituents is essentially due to the considerable advances in analytical methods in the course of the last half century.

2.3 RESEARCH DURING THE LAST HALF CENTURY

2.3.1 Essential Oil Preparation Techniques

2.3.1.1 Industrial Processes

The vast majority of essential oils are produced from plant material in which they occur by different kinds of distillation or by cold pressing in the case of the peel oils from citrus fruits.

In water or hydrodistillation, the chopped plant material is submerged and in direct contact with boiling water. In steam distillation, the steam is produced in a boiler separate of the still and blown through a pipe into the bottom of the still, where the plant material rests on a perforated tray or in a basket for quick removal after exhaustive extraction. In addition to the aforementioned distillation at atmospheric pressure, high-pressure steam distillation is most often applied in European and American field stills, and the applied increased temperature significantly reduces the time of distillation. The high-pressure steam-type distillation is often applied for peppermint, spearmint, lavandin, and the like. The condensed distillate, consisting of a mixture of water and oil, is usually separated in a so-called Florentine flask, a glass jar, or more recently in a receptacle made of stainless steel with one outlet near the base and another near the top. There, the distillate separates into two layers from which the oil and the water can be separately withdrawn. Generally, the process of steam distillation is the most widely accepted method for the production of essential oils on a large scale.

Expression or cold pressing is a process in which the oil glands within the peels of citrus fruits are mechanically crushed to release their content. There are several different processes used for the isolation of citrus oils; however, there are four major currently used processes. Those are pellatrice and sfumatrice—most often used in Italy—and the Brown peel shaver as well as the FMC extractor, which are used predominantly in North and South America. For more details, see, for example, Lawrence 1995. All these processes lead to products that are not entirely volatile because they may contain coumarins, plant pigments, and so on; however, they are nevertheless acknowledged as essential oils by the International Organization for Standardization, the different pharmacopoeias, and so on.

In contrast, extracts obtained by solvent extraction with different organic solvents, with liquid carbon dioxide or by supercritical fluid extraction (SFE) may not be considered as true essential oils; however, they possess most often aroma profiles that are almost identical to the raw material from which they have been extracted. They are therefore often used in the flavor and fragrance industry and in addition in food industry, if the chosen solvents are acceptable for food and do not leave any harmful residue in food products.

2.3.1.2 Laboratory-Scale Techniques

The following techniques are used mainly for trapping small amounts of volatiles from aromatic plants in research laboratories and partly for determination of the essential oil content in plant material.

The most often used device is the circulatory distillation apparatus, basing on the publication of Clevenger in 1928 and which has later found various modifications. One of those modified apparatus described by Cocking and Middleton (1935) has been introduced in the European pharmacopoeia and several other pharmacopoeias. This device consists of a heated round-bottom flask into which the chopped plant material and water are placed and which is connected to a vertical condenser and a graduated tube, for the volumetric determination of the oil. At the bottom of the tube, a three-way valve permits to direct the water back to the flask, since it is a continuous closed-circuit distillation device, and at the end of the distillation process to separate the essential oil from the water phase for further investigations. The length of distillation depends on the plant material to be investigated; however, it is usually fixed to 3–4 h. For the volumetric determination of the essential oil content in plants according to most of the pharmacopoeias, a certain amount of xylene—usually 0.5 mL—has to be placed over the water before running distillation to separate even small droplets of essential oil during distillation from the water. The volume of essential oil can be determined in the graduated tube after subtracting the volume of the applied xylene.

Improved constructions with regard to the cooling system of the aforementioned distillation apparatus have been published by Stahl (1953) and Sprecher (1963) and, in publications of Kaiser and Lang (1951) and Mechler and Kovar (1977), various apparatus used for the determination of essential oils in plant material are discussed and depicted.

A further improvement was the development of a simultaneous distillation–solvent extraction device by Likens and Nickerson in 1964 (see Nickerson and Likens, 1966). The device permits continuous concentration of volatiles during hydrodistillation in one step using a closed-circuit distillation system. The water distillate is continuously extracted with a small amount of an organic- and water-immiscible solvent. Although there are two versions described, one for high-density and one for low-density solvents, the high-density solvent version using dichloromethane is mostly applied in essential oil research. It has found numerous applications, and several modified versions including different microdistillation devices have been described (e.g., Bicchi et al., 1987; Chaintreau, 2001).

A sample preparation technique basing on Soxhlet extraction in a pressurized container using liquid carbon dioxide as extractant has been published by Jennings (1979). This device produces solvent-free extracts especially suitable for high-resolution gas chromatography (GC). As a less time-consuming alternative, the application of microwave-assisted extraction has been proposed by several researchers, for example, by Craveiro et al. (1989), using a round-bottom flask containing the fresh plant material. This flask was placed into a microwave oven and passed by a flow of air. The oven was heated for 5 min and the obtained mixture of water and oil collected in a small and cooled flask. After extraction with dichloromethane, the solution was submitted to GC–mass spectrometry (GC-MS) analysis. The obtained analytical results have been compared with the results obtained by conventional distillation and exhibited no qualitative differences; however, the percentages of the individual components varied significantly. A different approach yielding solvent-free extracts from aromatic herbs by means of microwave heating has been presented by Lucchesi et al. (2004). The potential of the applied technique has been compared with conventional hydrodistillation showing substantially higher amounts of oxygenated compounds at the expense of monoterpene hydrocarbons.

2.3.1.3 Microsampling Techniques

2.3.1.3.1 Microdistillation

Preparation of very small amounts of essential oils may be necessary if only very small amounts of plant material are available and can be fundamental in chemotaxonomic investigations and control analysis but also for medicinal and spice plant breeding. In the past, numerous attempts have been made to minimize conventional distillation devices. As an example, the modified Marcusson device may be quoted (Bicchi et al., 1983) by which 0.2–3 g plant material suspended in 50 mL water can be distilled and collected in 100 μL analytical grade pentane or hexane. The analytical results proved to be identical with those obtained by conventional distillation.

Microversions of the distillation–extraction apparatus, described by Likens and Nickerson, have also been developed as well for high-density (Godefroot et al., 1981) and low-density solvents (Godefroot et al., 1982). The main advantage of these techniques is that no further enrichment by evaporation is required for subsequent gas chromatographic investigation.

A different approach has been presented by Gießelmann and Kubeczka (1993) and Kubeczka and Gießelmann (1995). By means of a new developed micro-hydrodistillation device, the volatile constituents of very small amounts of plant material have been separated. The microscale hydrodistillation of the sample is performed using a 20 mL crimp-cap glass vial with a Teflon®-lined rubber septum containing 10 mL water and 200–250 mg of the material to be investigated. This vial, which is placed in a heating block, is connected with a cooled receiver vial by a 0.32 mm ID fused silica capillary. By temperature-programmed heating of the sample vial, the water and the volatile constituents are vaporized and passed through the capillary into the cooled receiver vial. There, the volatiles as well as water are condensed and the essential oil collected in pentane for further analysis. The received analytical results have been compared to results from identical samples obtained by conventional hydrodistillation showing a good correlation of the qualitative and quantitative composition. Further applications with the commercially available Eppendorf MicroDistiller® have been published in several papers, for example, by Briechle et al. (1997) and Baser et al. (2001).

A simple device for rapid extraction of volatiles from natural plant drugs and the direct transfer of these substances to the starting point of a thin-layer chromatographic plate has been described by Stahl (1969a) and in his subsequent publications. A small amount of the sample (ca. 100 mg) is introduced into a glass cartridge with a conical tip together with 100 mg silica gel, containing 20% of water, and heated rapidly in a heating block for a short time at a preset temperature. The tip of the glass tube projects ca. 1 mm from the furnace and points to the starting point of the thin-layer plate, which is positioned 1 mm in front of the tip. Before introducing the glass tube, it is sealed with a silicone rubber membrane. This simple technique has proven useful for many years in numerous investigations, especially in quality control, identification of plant drugs, and rapid screening of chemical races. In addition to the aforementioned micro-hydrodistillation with the so-called TAS procedure (T, thermomicro and transfer; A, application; S, substance), several further applications, for example, in structure elucidation of isolated natural compounds such as zinc dust distillation, sulfur and selenium dehydrogenation, and catalytic dehydrogenation with palladium, have been described in the microgram range (Stahl, 1976).

2.3.1.3.2 Direct Sampling from Secretory Structures

The investigation of the essential oils by direct sampling from secretory glands is of fundamental importance in studying the true essential oil composition of aromatic plants, since the usual applied techniques such as hydrodistillation and extraction are known to produce in some cases several artifacts. Therefore, only direct sampling from secretory cavities and glandular trichomes and properly performed successive analysis may furnish reliable results. One of the first investigations with a kind of direct sampling has been performed by Hefendehl (1966), who isolated the glandular hairs from the surfaces of *Mentha piperita* and *Mentha aquatica* leaves by means of a thin film of polyvinyl alcohol, which was removed after drying and extracted with diethyl ether. The composition of this product was in good agreement with the essential oils obtained by hydrodistillation. In contrast to these results, Malingré et al. (1969) observed some qualitative differences in the course of their study on *M. aquatica* leaves after isolation of the essential oil from individual glandular hairs by means of a micromanipulator and a stereomicroscope. In the same year, Amelunxen et al. (1969) published results on *M. piperita*, who separately isolated glandular hairs and glandular trichomes with glass capillaries. They found identical qualitative composition of the oil in both types of hairs, but differing concentrations of the individual components. Further studies have been performed by Henderson et al. (1970) on *Pogostemon cablin* leaves and by Fischer et al. (1987) on *Majorana hortensis* leaves. In the latter study, significant differences regarding the oil composition of the hydrodistilled oil and the oil extracted by means of glass capillaries from the trichomes were

observed. Their final conclusion was that the analysis of the respective essential oil is mainly an analysis of artifacts, formed during distillation, and the gas chromatographic analysis. Even if the investigations are performed very carefully and the successive GC has been performed by cold-on-column injection to avoid thermal stress in the injection port, significant differences of the GC pattern of directly sampled oils versus the microdistilled samples have been observed in several cases (Bicchi et al., 1985).

2.3.1.3.3 HS Techniques

Headspace (HS) analysis has become one of the very frequently used sampling techniques in the investigation of aromatic plants, fragrances, and spices. It is a means of separating the volatiles from a liquid or solid prior to gas chromatographic analysis and is preferably used for samples that cannot be directly injected into a gas chromatograph. The applied techniques are usually classified according to the different sampling principles in static HS analysis and dynamic HS analysis.

2.3.1.3.3.1 Static HS Methods In static HS analysis, the liquid or solid sample is placed into a vial, which is heated to a predetermined temperature after sealing. After the sample has reached equilibrium with its vapor (in equilibrium, the distribution of the analytes between the two phases depends on their partition coefficients at the preselected temperature, the time, and the pressure), an aliquot of the vapor phase can be withdrawn with a gas-tight syringe and subjected to gas chromatographic analysis. A simple method for the HS investigation of herbs and spices was described by Chialva et al. (1982), using a blender equipped with a special gas-tight valve. After grinding the herb and until thermodynamic equilibrium is reached, the HS sample can be withdrawn through the valve and injected into a gas chromatograph. Eight of the obtained capillary gas chromatograms are depicted in the paper of Chialva and compared with those of the respective essential oils exhibiting significant higher amounts of the more volatile oil constituents. However, one of the major problems with static HS analyses is the need for sample enrichment with regard to trace components. Therefore, a concentration step such as cryogenic trapping, liquid absorption, or adsorption on a suitable solid has to be inserted for volatiles occurring only in small amounts. A versatile and often-used technique in the last decade is solid-phase microextraction (SPME) for sampling volatiles, which will be discussed in more detail in a separate paragraph. Since different other trapping procedures are a fundamental prerequisite for dynamic HS methods, they will be considered in the succeeding text. A comprehensive treatment of the theoretical basis of static HS analysis including numerous applications has been published by Kolb and Ettre (1997, 2006).

2.3.1.3.3.2 Dynamic HS Methods The sensitivity of HS analysis can be improved considerably by stripping the volatiles from the material to be investigated with a stream of purified air or inert gas and trapping the released compounds. However, care has to be taken if grinded plant material has to be investigated, since disruption of tissues may initiate enzymatic reactions that may lead to formation of volatile artifacts. After stripping the plant material with gas in a closed vessel, the released volatile compounds are passed through a trap to collect and enrich the sample. This must be done because sample injection of fairly large sample volumes results in band broadening causing peak distortion and poor resolution. The following three techniques are advisable for collecting the highly diluted volatile sample according to Schaefer (1981) and Schreier (1984) with numerous references.

Cryogenic trapping can be achieved by passing the gas containing the stripped volatiles through a cooled vessel or a capillary in which the volatile compounds are condensed (Kolb and Liebhardt, 1986). The most convenient way for trapping the volatiles is to utilize part of the capillary column as a cryogenic trap. A simple device for cryofocusing of HS volatiles by using the first part of capillary column as a cryogenic trap has been shown in the aforementioned reference inclusive of a discussion of the theoretical background of cryogenic trapping. A similar on-column cold trapping device, suitable for extended period vapor sampling, has been published by Jennings (1981).

A different approach can be used if large volumes of stripped volatiles have to be trapped using collection in organic liquid phases. In this case, the volatiles distribute between the gas and the liquid, and efficient collection will be achieved, if the distribution factor *K* is favorable for solving the stripped compounds in the liquid. A serious drawback, however, is the necessity to concentrate the obtained solution prior to GC with the risk to lose highly volatile compounds. This can be overcome if a short-packed GC column is used containing a solid support coated with a suitable liquid. Novak et al. (1965) have used Celite coated with 30% silicone elastomer E-301 and the absorbed compounds were introduced into a gas chromatograph after thermal desorption. Coating with 15% silicone rubber SE 30 has been successfully used by Kubeczka (1967) with a similar device and the application of a wall-coated tubing with methyl silicone oil SF 96 has been described by Teranishi et al. (1972). A different technique has been used by Bergström (1973) and Bergström et al. (1980). They trapped the scent of flowers on Chromosorb® W coated with 10% silicon high-vacuum grease and filled a small portion of the sorbent containing the volatiles into a precolumn, which was placed in the splitless injection port of a gas chromatograph. There, the volatiles were desorbed under heating and flushed onto the GC column. In 1987, Bichi et al. applied up to 50 cm pieces of thick-film fused silica capillaries coated with a 15 μm dimethyl silicone film for trapping the volatiles in the atmosphere surrounding living plants. The plants under investigation were placed in a glass bell into which the trapping capillary was introduced through a rubber septum, while the other end of the capillary has been connected to pocket sampler. In order to trap even volatile monoterpene hydrocarbons, a capillary length of at least 50 cm and sample volume of maximum 100 mL have to be applied to avoid loss of components through breakthrough. The trapped compounds have been subsequently online thermally desorbed, cold trapped, and analyzed. Finally, a type of *enfleurage* especially designed for field experiments has been described by Joulain (1987) to trap the scents of freshly picked flowers. Around 100 g flowers were spread on the grid of a specially designed stainless steel device and passed by a stream of ambient air, supplied by an unheated portable air drier. The stripped volatiles are trapped on a layer of purified fat placed above the grid. After 2 h, the fat was collected and the volatiles recovered in the laboratory by means of vacuum distillation at low temperature.

With a third often applied procedure, the stripped volatiles from the HS of plant material and especially from flowers are passed through a tube filled with a solid adsorbent on which the volatile compounds are adsorbed. Common adsorbents most often used in investigations of plant volatiles are above all charcoal and different types of synthetic porous polymers. Activated charcoal is an adsorbent with a high adsorption capacity, thermal and chemical stability, and which is not deactivated by water, an important feature, if freshly collected plant material has to be investigated. The adsorbed volatiles can easily be recovered by elution with small amounts (10–50 μL) of carbon disulfide avoiding further concentration of the sample prior to GC analysis. The occasionally observed incomplete recovery of sample components after solvent extraction and artifact formation after thermal desorption has been largely solved by application of small amounts of special type of activated charcoal as described by Grob and Zürcher (1976). Numerous applications have been described using this special type of activated charcoal, for example, by Kaiser (1993) in a great number of field experiments on the scent of orchids. In addition to charcoal, the following synthetic porous polymers have been applied to collect volatile compounds from the HS from flowers and different other plant materials according to Schaefer (1981): Tenax® GC, different Porapak® types (e.g., Porapak P, Q, R, and T), and several Chromosorb types belonging to the 100 series. More recent developed adsorbents are the carbonaceous adsorbents such as Ambersorb®, Carboxen®, and Carbopak®, and their adsorbent properties lie between activated charcoal and the porous polymers. Especially the porous polymers have to be washed repeatedly, for example, with diethyl ether, and conditioned before use in a stream of oxygen-free nitrogen at 200°C–280°C, depending on the sort of adsorbent. The trapped components can be recovered either by thermal desorption or by solvent elution, and the recoveries can be different depending on the applied adsorbent (Cole, 1980). Another very important criterion for the selection of a suitable adsorbent for collecting HS samples is the breakthrough volume limiting the amount of gas passing through the trap.

A comprehensive review concerning HS gas chromatographic analysis of medicinal and aromatic plants and flowers with 137 references, covering the period from 1982 to 1988 has been published by Bicchi and Joulain in 1990, thoroughly describing and explaining the different methodological approaches and applications. Among other things, most of the important contributions of the Finnish research group of Hiltunen and coworkers on the HS of medicinal plants and the optimization of the HS parameters have been cited in the mentioned review.

2.3.1.3.4 Solid-Phase Microextraction

SPME is an easy-to-handle sampling technique, initially developed for the determination of volatile organic compounds in environmental samples (Arthur and Pawliszyn, 1990), and has gained, in the last years, acceptance in numerous fields and has been applied to the analysis of a wide range of analytes in various matrices. Sample preparation is based on sorption of analytes from a sample onto a coated fused silica fiber, which is mounted in a modified GC syringe. After introducing the coated fiber into a liquid or gaseous sample, the compounds to be analyzed are enriched according to their distribution coefficients and can be subsequently thermally desorbed from the coating after introducing the fiber into the hot injector of a gas chromatograph. The commercially available SPME device (Supelco Inc.) consists of a 1 cm length fused silica fiber of ca. 100 μm diameter coated on the outer surface with a stationary phase fixed to a stainless steel plunger and a holder that looks like a modified microliter syringe (Supelco, 2007). The fiber can be drawn into the syringe needle to prevent damage. To use the device, the needle is pierced through the septum that seals the sample vial. Then, the plunger is depressed lowering the coated fiber into the liquid sample or the HS above the sample. After sorption of the sample, which takes some minutes, the fiber has to be drawn back into the needle and withdrawn from the sample vial. By the same procedure, the fiber can be introduced into the gas chromatograph injector where the adsorbed substances are thermally desorbed and flushed by the carrier gas into the capillary GC column.

SPME fibers can be coated with polymer liquid (e.g., polydimethylsiloxane [PDMS]) or a mixed solid and liquid coating (e.g., Carboxen®/PDMS). The selectivity and capacity of the fiber coating can be adjusted by changing the phase type or thickness of the coating on the fiber according to the properties of the compounds to be analyzed. Commercially available are coatings of 7, 30, and 100 μm of PDMS, an 85 μm polyacrylate, and several mixed coatings for different polar components. The influence of fiber coatings on the recovery of plant volatiles was thoroughly investigated by Bicchi et al. (2000a,b). Details concerning the theory of SPME, technology, its application, and specific topics have been described by Pawliszyn (1997) and references cited therein. A number of different applications of SPME in the field of essential oil analysis have been presented by Kubeczka (1997a). An overview on publications of the period 2000–2005 with regard to HS-SPME has been recently published by Belliardo et al. (2006) covering the analysis of volatiles from aromatic and medicinal plants, selection of the most effective fibers and sampling conditions, and discussing its advantages and limitations. The most comprehensive collection of references with regard to the different application of SPME can be obtained from Supelco on CD.

2.3.1.3.5 Stir Bar Sorptive Extraction and HS Sorptive Extraction

Despite the indisputable simplicity and rapidity of SPME, its applicability is limited by the small amount of sorbent on the needle (<0.5 μL), and consequently SPME has no real opportunity to realize quantitative extraction. Parameters governing recovery of analytes from a sample are partitioning constants and the phase ratio between the sorbent and liquid or gaseous sample. Therefore, basing on theoretical considerations, a procedure for sorptive enrichment with the sensitivity of packed PDMS beds (Baltussen et al., 1997) has been developed for the extraction of aqueous samples using modified PDMS-coated stir bars (Baltussen et al., 1999).

The stir bars were incorporated into a narrow glass tube coated with a PDMS layer of 1 mm (corresponding to 55 μL for a 10 mm length) applicable to small sample volumes. Such stir bars are commercially available under the name "Twister" (Gerstel, Germany). After certain stirring time, the stir

bar has to be removed, introduced into a glass tube, and transferred to thermal desorption instrument. After desorption and cryofocusing within a cooled programmed temperature vaporization (PTV) injector, the volatiles were transferred onto the analytical GC column. Comparison of SPME and the aforementioned stir bar sorptive extraction (SBSE) technique using identical phases for both techniques exhibited striking differences in the recoveries, which has been attributed to ca. 100 times higher phase ratio in SBSE than in SPME. A comprehensive treatment of SBSE, discussion of the principle, the extraction procedure, and numerous applications was recently been published by David and Sandra (2007).

A further approach for sorptive enrichment of volatiles from the HS of aqueous or solid samples has been described by Tienpont et al. (2000), referred to as HS sorptive extraction (HSSE). This technique implies the sorption of volatiles into PDMS that is chemically bound on the surface of a glass rod support. The device consists of a ca. 5 cm length glass rod of 2 mm diameter and at the last centimeter of 1 mm diameter. This last part is covered with PDMS chemically bound to the glass surface. HS bars with 30, 50, and 100 mg PDMS are commercially available from Gerstel GmbH, Mülheim, Germany. After thermal conditioning at 300°C for 2 h, the glass bar was introduced into the HS of a closed 20 mL HS vial containing the sample to be investigated. After sampling for 45 min, the bar was put into a glass tube for thermal desorption, which was performed with a TDS-2 thermodesorption unit (Gerstel). After desorption and cryofocusing within a PTV injector, the volatiles were transferred onto the analytical GC column. As a result, HSSE exceeded largely the sensitivity attainable with SPME. Several examples referring to the application of HSSE in HS analysis of aromatic and medicinal plants inclusive of details of the sampling procedure were described by Bicchi et al. (2000a).

2.3.2 Chromatographic Separation Techniques

In the course of the last half century, a great number of techniques have been developed and applied to the analysis of essential oils. A part of them has been replaced nowadays by either more effective or easier-to-handle techniques, while other methods maintained their significance and have been permanently improved. Before going into detail, the analytical facilities in the sixties of the last century should be considered briefly. The methods available for the analysis of essential oils have been at that time (Table 2.1) thin-layer chromatography (TLC), various types of liquid column chromatography (LC), and already gas–liquid chromatography (GC). In addition, several spectroscopic techniques such as UV and IR spectroscopy, MS, and ^{1}H-NMR spectroscopy have been available. In the following years, several additional techniques were developed and applied to essential oils analysis, including high-performance liquid chromatography (HPLC); different

TABLE 2.1
Techniques Applied to the Analysis of Essential Oils

Chromatographic Techniques Including Two- and Multidimensional Techniques	Spectroscopic and Spectrometric Techniques	Hyphenated Techniques
TLC	UV	GC-MS
GC	IR	GC-UV
LC	MS	HPLC-GC
HPLC	^{1}H-NMR	SFE-GC
CCC	^{13}C-NMR	GC-FTIR
SFC	NIR	GC-AES
	Raman	HPLC-MS
		SFC-GC
		GC-FTIR-MS
		GC-IRMS
		HPLC-NMR

kinds of countercurrent chromatography (CCC); supercritical fluid chromatography (SFC), including multidimensional coupling techniques, C-13 NMR, near IR (NIR), and Raman spectroscopy; and a multitude of so-called hyphenated techniques, which means online couplings of chromatographic separation devices to spectrometers, yielding valuable structural information of the individual separated components that made their identification feasible.

2.3.2.1 Thin-Layer Chromatography

TLC was one of the first chromatographic techniques and has been used for many years for the analysis of essential oils. This method provided valuable information compared to simple measurements of chemical and physical values and has therefore been adopted as a standard laboratory method for characterization of essential oils in numerous pharmacopoeias. Fundamentals of TLC have been described by Geiss (1987) and in a comprehensive handbook by Stahl (1969b), in which numerous applications and examples on investigations of secondary plant metabolites inclusive of essential oils are given. More recently, the third edition of the handbook of TLC from Shema and Fried (2003) appeared. Further approaches in TLC have been the development of high-performance TLC (Kaiser, 1976) and the application of forced flow techniques such as overpressured layer chromatography and rotation planar chromatography described by Tyihák et al. (1979) and Nyiredy (2003).

In spite of its indisputable simplicity and rapidity, this technique is now largely obsolete for analyzing such complex mixtures like essential oils, due to its low resolution. However, for the rapid investigation of the essential oil pattern of chemical races or the differentiation of individual plant species, this method can still be successfully applied (Gaedcke and Steinhoff, 2000). In addition, silver nitrate and silver perchlorate impregnated layers have been used for the separation of olefinic compounds, especially sesquiterpene hydrocarbons (Prasad et al., 1947), and more recently for the isolation of individual sesquiterpenes (Saritas, 2000).

2.3.2.2 GC

However, the separation capability of GC exceeded all the other separation techniques, even if only packed columns have been used. The exiting evolution of this technique in the past can be impressively demonstrated with four examples of the gas chromatographic separation of the essential oil from rue (Kubeczka, 1981a), a medicinal and aromatic plant. This oil was separated by S. Bruno in 1961 into eight constituents and represented one of the first gas chromatographic analyses of that essential oil. Only a few years later in 1964, separation of the same oil has been improved using a Perkin Elmer gas chromatograph equipped with a 2 m packed column and a thermal conductivity detector (TCD) operated under isothermal conditions yielding 20 separated constituents. A further improvement of the separation of the rue oil was obtained after the introduction of temperature programming of the column oven, yielding approximately 80 constituents. The last significant improvements were a result of the development of high-resolution capillary columns and the sensitive flame ionization detector (FID) (Bicchi and Sandra, 1987). By means of a 50 m glass capillary with 0.25 mm ID, the rue oil could be separated into approximately 150 constituents, in 1981. However, the problems associated with the fragility of the glass capillaries and their cumbersome installation lessened the acknowledgment of this column types, despite their outstanding quality. This has changed since flexible fused silica capillaries became commercially available, which are nearly unbreakable in normal usage. In addition, by different cross-linking technologies, the problems associated with wall coating, especially with polar phases, have been overcome, so that all important types of stationary phases used in conventional GC have been commercially available. The most often used stationary phases for the analysis of essential oils have been, and are still today, the polar phases Carbowax® 20M (DB-Wax, Supelcowax-10, HP-20M, Innowax, etc.) and 14% cyanopropylphenyl−86% methyl polysiloxane (DB- 1701, SPB-1701, HP-1701, OV-1701, etc.) and the nonpolar phases PDMS (DB-1, SPB-1, HP-1 and HP-1 ms, CPSil-5 CB, OV-1, etc.) and 5% phenyl methyl polysiloxane (DB-5, SPB-5, HP-5, CPSil-8 CB, OV-5, SE-54, etc.). Besides different column diameters of 0.53, 0.32, 0.25, 0.10, and 0.05 mm ID, a variety of film thicknesses can be purchased. Increasing column diameter and film thickness of stationary phase increases the sample

capacity at the expense of separation efficiency. However, sample capacity has become important, particularly in trace analysis and with some hyphenated techniques such as GC–Fourier transform IR (GC-FTIR), in which a higher sample capacity is necessary when compared to GC-MS. On the other hand, the application of a narrow bore column with 100 μm ID and a film coating of 0.2 μm have been shown to be highly efficient and theoretical plate numbers of approximately 250,000 were received with a 25 m capillary (Lancas et al., 1988). The most common detector in GC is the FID because of its high sensitivity toward organic compounds. The universal applicable TCD is nowadays used only for fixed-gas detection because of its very low sensitivity as compared to FID, and cannot be used in capillary GC. Nitrogen-containing compounds can be selectively detected with the aid of the selective nitrogen–phosphorus detector and chlorinated compounds by the selective and very sensitive electron-capture detector, which is often used in the analysis of pesticides. Oxygen-containing compounds have been selectively detected with special O-FID analyzer even in very complex samples, which was primarily employed to the analysis of oxygenated compounds in gasoline, utilized as fuel-blending agents (Schneider et al., 1982). The oxygen selectivity of the FID is obtained by two online postcolumn reactions: first, a cracking reaction forming carbon monoxide, which is reduced in a second reactor yielding equimolar quantities of methane, which can be sensitively detected by the FID. Since in total each oxygen atom is converted to one molecule methane, the FID response is proportional to the amount of oxygen in the respective molecule. Application of the O-FID to the analysis of essential oils has been presented by Kubeczka (1991). However, conventional GC using fused silica capillaries with different stationary phases, including chiral phases, and the sensitive FID, is up to now the prime technique for the analysis of essential oils.

2.3.2.2.1 Fast and Ultrafast GC

Due to the demand for faster GC separations in routine work in the field of GC of essential oils, the development of fast and ultrafast GC seems worthy to be mentioned. The various approaches for fast GC have been reviewed in 1999 (Cramers et al., 1999). The most effective way to speed up GC separation without losing separation efficiency is to use shorter columns with narrow inner diameter and thinner coatings, higher carrier gas flow rates, and accelerated temperature ramps. In Figure 2.1, the conventional and fast GC separation of lime oil is shown, indicating virtually the same separation efficiency in the fast GC and a reduction in time from approximately 60 to 13 min (Mondello et al., 2000).

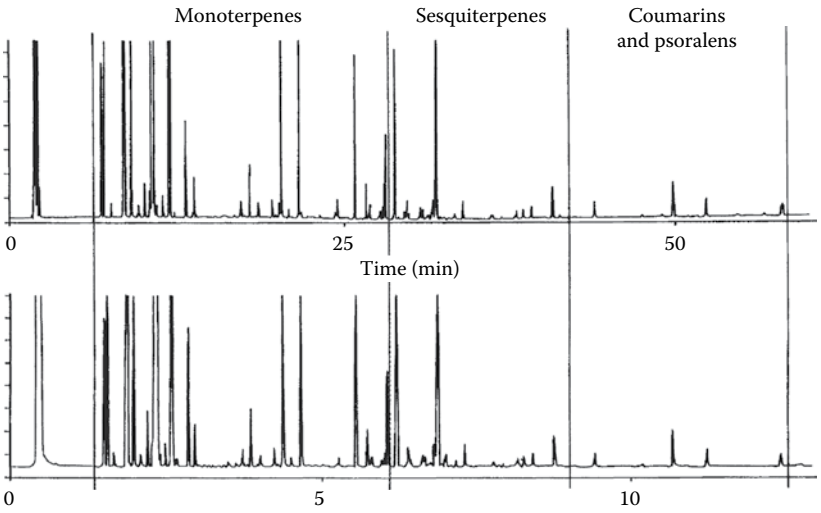

FIGURE 2.1 Comparison of conventional and fast GC separation of lime oil. (From Mondello, L. et al., *LC-GC Eur.*, 13, 495, 2000. With permission.)

TABLE 2.2
Conditions of Conventional, Fast, and Ultrafast GC

	Conventional GC	Fast GC	Ultrafast GC
Column	30 m	10 m	10–15 m
	0.25 mm ID	0.1 mm ID	0.1 mm ID
	0.25 μm film	0.1 μm film	0.1 μm film
Temperature program	50°C–350°C	50°C–350°C	45°C–325°C
	3°C/min	14°C/min	45–200°C/min
Carrier gas	H_2	H_2	H_2
	$u = 36$ cm/s	$u = 57$ cm/s	$u = 120$ cm/s
Sampling frequency	10 Hz	20–50 Hz	50–250 Hz

An ultrafast GC separation of the essential oil from lime with an outstanding reduction of time was recently achieved (Mondello et al., 2004) using a 5 m capillary with 50 μm ID and a film thickness of 0.05 μm operated with a high carrier gas velocity of 120 cm/min and an accelerated three-stage temperature program. The analysis of the essential oil was obtained in approximately 90 s, which equates to a speed gain of approximately 33 times in comparison with the conventional GC separation. However, such a separation cannot be performed with conventional GC instruments. In addition, the mass spectrometric identification of the separated components could only be achieved by coupling GC to a time-of-flight mass spectrometer. In Table 2.2, the separation parameters of conventional, fast, and ultrafast GC separation are given, indicating clearly the relatively low requirements for fast GC, while ultrafast separations can only be realized with modern GC instruments and need a significant higher employment.

2.3.2.2.2 Chiral GC

Besides fast and ultrafast GC separations, one of the most important developments in GC has been the introduction of enantioselective capillary columns in the past with high separation efficiency, so that a great number of chiral substances including many essential oil constituents could be separated and identified. The different approaches of gas chromatographic separation of chiral compounds are briefly summarized in Table 2.3. In the mid-1960s, Gil-Av published results with chiral diamide stationary phases for gas chromatographic separation of chiral compounds, which interacted with the analytes by hydrogen bonding forces (Gil-Av et al., 1965). The ability to separate enantiomers using these phases was therefore limited to substrates with hydrogen bonding donor or acceptor functions.

Diastereomeric association between chiral molecules and chiral transition metal complexes was first described by Schurig (1977). Since hydrogen bonding interaction is not essential for chiral recognition in such a system, a number of compounds could be separated, but this method was limited by the nonsufficient thermal stability of the applied metal complexes.

In 1988 König, as well as Schurig, described the use of cyclodextrin derivatives that act enantioselectively by host–guest interaction by partial intrusion of enantiomers into the cyclodextrin

TABLE 2.3
Different Approaches of Enantioselective GC

1. Chiral diamide stationary phases (Gil-Av et al., 1965)
   Hydrogen bonding interaction
2. Chiral transition metal complexation (Schurig, 1977)
   Complexation gas chromatography
3. Cyclodextrin derivatives (König et al, 1988a,b,c and Schurig and Nowotny, 1988)
   Host–guest interaction, inclusion gas chromatography

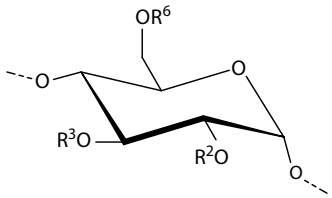

FIGURE 2.2 α-Glucose unit of a cyclodextrin.

cavity. They are cyclic α-(1–4)-bounded glucose oligomers with six-, seven-, or eight-glucose units, which can be prepared by enzymatic degradation of starch with specific cyclodextrin glucosyl transferases from different bacterial strains, yielding α-, β-, and γ-cyclodextrins, and are commercially available. Due to the significant lower reactivity of the 3-hydroxygroups of cyclodextrins, this position can be selectively acylated after alkylation of the two and six positions (Figure 2.2), yielding several nonpolar cyclodextrin derivatives, which are liquid or waxy at room temperature and which proved very useful for gas chromatographic applications.

König and coworkers reported their first results in 1988 with per-*O*-pentylated and selectively 3-*O*-acylated-2,6-di-*O*-pentylated α-, β-, and γ-cyclodextrins, which are highly stable, soluble in nonpolar solvents, and which possess a high enantioselectivity toward many chiral compounds. In the following years, a number of further cyclodextrin derivatives have been synthesized and tested by several groups, allowing the separation of a wide range of chiral compounds, especially due to the improved thermal stability (Table 2.4) (König et al., 1988a,b,c). With the application of 2,3-pentyl-6-methyl-β- and -γ-cyclodextrin as stationary phases, all monoterpene hydrocarbons commonly occurring in essential oils could be separated (König et al., 1992a). The reason for application of two different columns with complementary properties was that on one column not all enantiomers were satisfactorily resolved. Thus, the simultaneous use of these two columns provided a maximum of information and reliability in peak assignment (König et al., 1992b).

After successful application of enantioselective GC to the analysis of enantiomeric composition of monoterpenoids in many essential oils (e.g., Werkhoff et al., 1993; Bicchi et al., 1995; and references cited therein), the studies have been extended to the sesquiterpene fraction. Standard mixtures of known enantiomeric composition were prepared by isolation of individual enantiomers from numerous essential oils by preparative GC and by preparative enantioselective GC. A gas chromatographic separation of a series of isolated or prepared sesquiterpene hydrocarbon enantiomers, showing

TABLE 2.4
Important Cyclodextrin Derivatives

Research Group	Year	Cyclodextrin Derivative
Schurig and Novotny	1988	Per-*O*-methyl-β-CD
König et al.	1988c	Per-*O*-pentyl-(α,β,γ)-CD
König et al.	1988b	3-*O*-acetyl-2,6,-di-*O*-pentyl-(α,β,γ)-CD
König et al.	1989	3-*O*-butyryl-2,6-di-*O*-pentyl-(α,β)-CD
König et al.	1990	6-*O*-methyl-2,3-di-*O*-pentyl-γ-CD
Köng et al.	1990	2,6-Di-*O*-methyl-3-*O*-pentyl-(α,γ)-CD
Dietrich et al.	1992b	2,3-Di-*O*-acetyl-6-*O*-*tert*-butyl-dimethysilyl-β-CD
Dietrich et al.	1992a	2,3-Di-*O*-methyl-6-*O*-*tert*-butyl-dimethylsilyl-(β,γ)-CD
Bicchi et al.	1996	2,3-Di-*O*-ethyl-6-*O*-*tert*-butyl-dimethylsilyl-(β,γ)-CD
Takahisa and Engel	2005a	2,3-Di-*O*-methoxymethyl-6-*O*-*tert*-butyl-dimethylsilyl-β-CD
Takahisa and Engel	2005b	2,3-Di-*O*-methoxymethyl-6-*O*-*tert*-butyl-dimethylsilyl-γ-CD

the separation of 12 commonly occurring sesquiterpene hydrocarbons on a 2,6-methyl-3-pentyl-β-cyclodextrin capillary column has been presented by König et al. (1995). Further investigations on sesquiterpenes have been published by König et al. (1994). However, due to the complexity of the sesquiterpene pattern in many essential oils, it is often impossible to perform directly an enantioselective analysis by coinjection with standard samples on a capillary column with a chiral stationary phase alone. Therefore, in many cases 2D GC had to be performed.

2.3.2.2.3 Two-Dimensional GC

After preseparation of the oil on a nonchiral stationary phase, the peaks of interest have to be transferred to a second capillary column coated with a chiral phase, a technique usually referred to as "heart cutting." In the simplest case, two GC capillaries with different selectivities are serially connected, and the portion of unresolved components from the effluent of the first column is directed into a second column, for example, a capillary with a chiral coating. The basic arrangement used in 2D GC (GC-GC) is shown in Figure 2.3. By means of a valve, the individual fractions of interest eluting from the first column are directed to the second, chiral column, while the rest of the sample may be discarded. With this heart-cutting technique, many separations of chiral oil constituents have been performed in the past. As an example, the investigation of the chiral sesquiterpene hydrocarbon germacrene D shall be mentioned (Kubeczka, 1996), which was found to be a main constituent of the essential oil from the flowering herb from *Solidago canadensis*. The enantioselective investigation of the germacrene-D fraction from a GC run using a nonchiral DB-Wax capillary transferred to a 2,6-methyl-3-pentyl-β-cyclodextrin capillary exhibited the presence of both enantiomers. This is worthy to be mentioned, since in most of other germacrene D containing higher plants nearly exclusively the (–)-enantiomer can be found.

The previously mentioned 2D GC design, however, in which a valve is used to direct the portion of desired effluent from the first into the second column, has obviously several shortcomings. The sample comes into contact with the metal surface of the valve body, the pressure drop of both connected columns may be significant, and the use of only one-column oven does not permit to adjust the temperature for both columns properly. Therefore, one of the best approaches to overcome these limitations has been realized by a commercially available two-column oven instrument using a Deans-type pressure balancing interface between the two columns called a "live-T connection" (Figure 2.4) providing considerable flexibility (Hener, 1990). By means of that instrument, the enantiomeric composition of several essential oils has been investigated very successfully. As an example, the investigation of the essential oil from *Lavandula angustifolia* shall be mentioned (Kreis

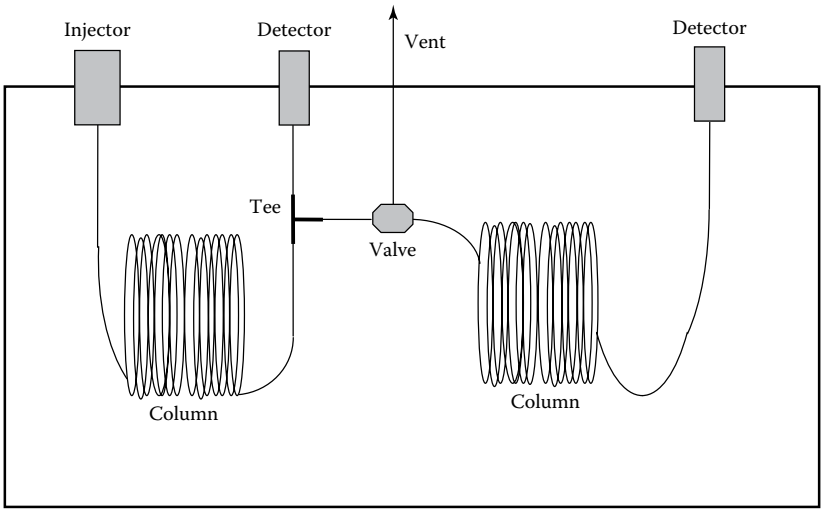

FIGURE 2.3 Basic arrangement used in 2D GC.

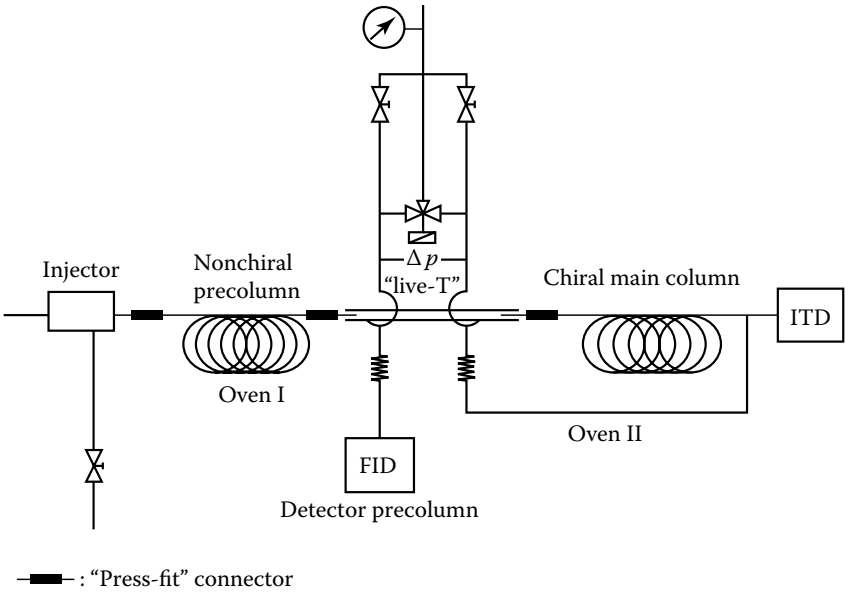

FIGURE 2.4 Scheme of enantioselective multidimensional GC with "live-T" column switching. (From Hener, U., Chirale Aromastoffe—Beiträge zur Struktur, Wirkung und Analytik, Dissertation, Goethe-University of Frankfurt/Main, Frankfurt, Germany, 1990. With permission.)

and Mosandl, 1992) showing the simultaneous stereoanalysis of a mixture of chiral compounds, which can be found in lavender oils, using the column combination Carbowax 20M as the precolumn and 2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin as the main column. All the unresolved enantiomeric pairs from the precolumn could be well separated after transferring them to the chiral main column in a single run. As a result, it was found that most of the characteristic and genuine chiral constituents of lavender oil exhibit a high enantiomeric purity.

A different and inexpensive approach for transferring individual GC peaks onto a second column has been presented by Kubeczka (1997a , using an SPME device. The highly diluted organic vapor of a fraction eluting from a GC capillary in the carrier gas flow has been absorbed on a coated SPME fiber and introduced onto a second capillary. As could be demonstrated, no modification of the gas chromatograph had to be performed to realize that approach. The eluting fractions were sampled after shutting the valves of the air, of hydrogen and the makeup gas if applied. In order to minimize the volume of the detector to avoid dilution of the eluting fraction and to direct the gas flow to the fiber surface, a capillary glass tubing of 1.5 mm ID was inserted into the FID and fixed and tightened by an O-ring (Figure 2.5). At the beginning of peak elution, controlled only by time, a 100 μm PDMS fiber was introduced into the mounted glass capillary tubing and withdrawn at the end of peak elution. Afterward, the fiber within the needle was introduced into the injector of a second capillary column with a chiral stationary phase. Two examples concerning the investigation of bergamot oil have been shown. At first, the analysis of an authentic sample of bergamot oil, containing chiral linalool, and the respective chiral actetate is carried out. Both components were cut separately and transferred to an enantioselective cyclodextrin Lipodex® E capillary. The chromatograms clearly have shown that the authentic bergamot oil contains nearly exclusively the (−)-enantiomers of linalool and linalyl acetate, while the respective (+)-enantiomers could only be detected as traces. In contrast to the authentic sample, a commercial sample of bergamot oil, which was analyzed under the same conditions, exhibited the presence of significant amounts of both enantiomers of linalool and linalyl acetate indicating a falsification by admixing the respective racemic alcohol and ester.

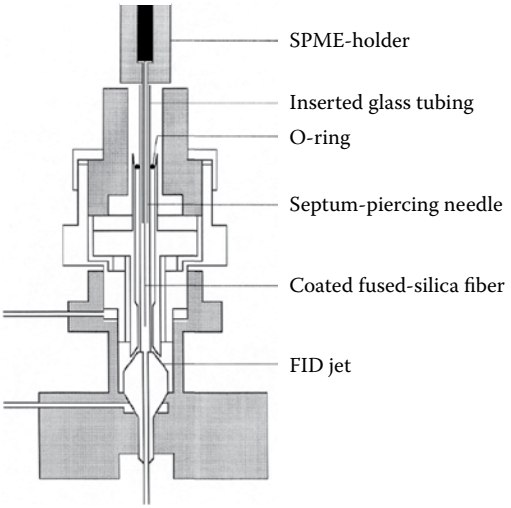

FIGURE 2.5 Cross section of an FID of an HP 5890 gas chromatograph with an inserted SPME fiber. (From Kubeczka, K.-H. *Essential Oil Symposium Proceedings*, 1997b, p. 145. With permission.)

2.3.2.2.4 Comprehensive Multidimensional GC

One of the most powerful separation techniques that has been recently applied to the investigation of essential oils is the so-called comprehensive multidimensional GC (GC × GC). This technique is a true multidimensional GC (MDGC) since it combines two directly coupled columns and importantly is able to subject the entire sample to simultaneous two-column separation. Using that technique, the need to select heart cuts, as used in conventional MDGC, is no longer required. Since components now are retained in two different columns, the net capacity is the product of the capacities of the two applied columns increasing considerably the resolution of the total system. Details regarding that technique will be given in Chapter 7.

2.3.2.3 Liquid Column Chromatography

The different types of LC have been mostly used in preparative or semipreparative scale for preseparation of essential oils or for isolation of individual oil constituents for structure elucidation with spectroscopic methods and were rarely used at that time as an analytical separation tool alone, because GC plays a central role in the study of essential oils.

2.3.2.3.1 Preseparation of Essential Oils

A different approach besides 2D GC, which has often been used in the past to overcome peak overlapping in a single GC run of an essential oil, has been preseparation of the oil with LC. The most common method of fractionation is the separation of hydrocarbons from the oxygenated terpenoids according to Miller and Kirchner (1952), using silica gel as an adsorbent. After elution of the nonpolar components from the column with pentane or hexane, the more polar oxygen-containing constituents are eluted in order of increasing polarity after applying more and more polar eluents.

A very simple and standardized fractionation in terms of speed and simplicity has been published by Kubeczka (1973) using dry-column chromatography. The procedure, which has been proved useful in numerous experiments for prefractionation of an essential oil, allows a preseparation into five fractions of increasing polarity. The preseparation of an essential oil into oxygenated constituents, monoterpene hydrocarbons, and sesquiterpene hydrocarbons, which is—depending on the oil composition—sometimes of higher practical use, can be performed successfully using reversed-phase RP-18 HPLC (Schwanbeck et al., 1982). The HPLC was operated on a semipreparative scale by stepwise elution with methanol–water 82.5:17.5 (solvent A) and pure methanol (solvent B).

The elution order of the investigated oil was according to decreasing polarity of the components and within the group of hydrocarbons to increasing molecular weight. Fraction 1 contained all oxygenated mono- and sesquiterpenoids, fraction 2 the monoterpene hydrocarbons, and fraction 3—eluted with pure methanol—the sesquiterpene hydrocarbons. A further alternative to the mentioned separation techniques is flash chromatography, initially developed by Still et al. (1978), which has often been used as a rapid form of preparative LC based on a gas- or air pressure–driven short-column chromatography. This technique, optimized for rapid separation of quantities typically in the range of 0.5–2.0 g, uses dry-packed silica gel in an appropriate column. The separation of the sample generally takes only 5–10 min and can be performed with inexpensive laboratory equipment. However, impurities and active sites on dried silica gel were found to be responsible for isomerization of a number of oil constituents. After deactivation of the dried silica gel by adding 5% water, isomerization processes could be avoided (Scheffer et al., 1976). A different approach using HPLC on silica gel and isocratic elution with a ternary solvent system for the separation of essential oils has been published by Chamblee et al. (1985). In contrast to the aforementioned commonly used offline pretreatment of a sample, the coupling of two or more chromatographic systems in an online mode offers advantages of ease of automation and usually of a shorter analysis time.

2.3.2.3.2 High-Performance Liquid Column Chromatography

The good separations obtained by GC have delayed the application of HPLC to the analysis of essential oils; however, HPLC analysis offers some advantages, if GC analysis of thermolabile compounds is difficult to achieve. Restricting factors for application of HPLC for analyses of terpenoids are the limitations inherent in the commonly available detectors and the relatively small range of k' values of liquid chromatographic systems. Since temperature is an important factor that controls k' values, separation of terpene hydrocarbons was performed at −15°C using a silica gel column and *n*-pentane as a mobile phase. Monitoring has been achieved with UV detection at 220 nm. Under these conditions, mixtures of commonly occurring mono- and sesquiterpene hydrocarbons could be well separated (Schwanbeck and Kubeczka, 1979; Kubeczka, 1981b). However, the silica gel had to be deactivated by adding 4.8% water prior to separation to avoid irreversible adsorption or alteration of the sample. The investigation of different essential oils by HPLC already has been described in the seventies of the last century (e.g., Komae and Hayashi, 1975; Ross, 1976; Wulf et al., 1978; McKone, 1979; Scott and Kucera, 1979). In the last publication, the authors have used a rather long microbore packed column, which had several hundred thousand theoretical plates. Besides relatively expensive equipment, the HPLC chromatogram of an essential oil, separated on such a column, could only be obtained at the expense of long analysis time. The mentioned separation needed about 20 h and may be only of little value in practical applications.

More recent papers with regard to HPLC separation of essential oils were published, for example, by Debrunner et al. (1995), Bos et al. (1996), and Frérot and Decorzant (2004), and applications using silver ion–impregnated sorbents have been presented by Pettei et al. (1977), Morita et al. (1983), Friedel and Matusch (1987), and van Beek et al. (1994). The literature on the use and theory of silver complexation chromatography has been reviewed by van Beek and Subrtova (1995). HPLC has also been used to separate thermally labile terpenoids at low temperature by Beyer et al. (1986), showing the temperature dependence of the separation efficiency. The investigation of an essential oil fraction from *Cistus ladanifer* using RP-18 reversed-phase HPLC at ambient temperature and an acetonitrile–water gradient was published by Strack et al. (1980). Comparison of the obtained HPLC chromatogram with the respective GC run exhibits a relatively good HPLC separation in the range of sesqui- and diterpenes, while the monoterpenes exhibited, as expected, a significant better resolution by GC. The enantiomeric separation of sesquiterpenes by HPLC with a chiral stationary phase has recently been shown by Nishii et al. (1997), using a Chiralcel® OD column.

2.3.2.4 Supercritical Fluid Chromatography

Supercritical fluids are highly compressed gases above their critical temperature and critical pressure point, representing a hybrid state between a liquid and a gas, which have physical properties

intermediate between liquid and gas phases. The diffusion coefficient of a fluid is about two orders of magnitude larger and the viscosity is two orders of magnitude lower than the corresponding properties of a liquid. On the other hand, a supercritical fluid has a significant higher density than a gas. The commonly used carbon dioxide as a mobile phase, however, exhibits a low polarity (comparable to pentane or hexane), limiting the solubility of polar compounds, a problem that has been solved by adding small amounts of polar solvents, for example, methanol or ethanol, to increase mobile-phase polarity, thus permitting separations of more polar compounds (Chester and Innis, 1986). A further strength of SFC lies in the variety of detection systems that can be applied. The intermediate features of SFC between GC and LC can be profitable when used in a variety of detection systems, which can be classified in *LC-* and *GC-like* detectors. In the first case, measurement takes place directly in the supercritical medium or in the liquid phase, whereas GC-like detection proceeds after a decompression stage.

Capillary SFC using carbon dioxide as mobile phase and a FID as detector has been applied to the analysis of several essential oils and seemed to give more reliable quantification than GC, especially for oxygenated compounds. However, the separation efficiency of GC for monoterpene hydrocarbons was, as expected, better than that of SFC. Manninen et al. (1990) published a comparison of a capillary GC versus a chromatogram obtained by capillary SFC from a linalool–methyl chavicol basil oil chemotype exhibiting a fairly good separation by SFC.

2.3.2.5 Countercurrent Chromatography

CCC is according to Conway (1989) a form of liquid–liquid partition chromatography, in which centrifugal or gravitational forces are employed to maintain one liquid phase in a coil or train of chambers stationary, while a stream of a second, immiscible phase is passed through the system in contact with the stationary liquid phase. Retention of the individual components of the sample to be analyzed depends only on their partition coefficients and the volume ratio of the two applied liquid phases. Since there is no porous support, adsorption and catalytic effects encountered with solid supports are avoided.

2.3.2.5.1 Droplet Countercurrent Chromatography

One form of CCC, which has been sporadically applied to separate essential oils into fractions or in the ideal case into individual pure components, is droplet countercurrent chromatography (DCCC). The device, which has been developed by Tanimura et al. (1970), consists of 300–600 glass tubes, which are connected to each other in series with Teflon tubing and filled with a stationary liquid. Separation is achieved by passing droplets of the mobile phase through the columns, thus distributing mixture components at different ratios leading to their separation. With the development of a water-free solvent system, separation of essential oils could be achieved (Becker et al., 1981, 1982). Along with the separation of essential oils, the method allows the concentration of minor components, since relatively large samples can be separated in one analytical run (Kubeczka, 1985).

2.3.2.5.2 Rotation Locular Countercurrent Chromatography

The rotation locular countercurrent chromatography (RLCC) apparatus (Rikakikai Co., Tokyo, Japan) consists of 16 concentrically arranged and serially connected glass tubes. These tubes are divided by Teflon disks with a small hole in the center, thus creating small compartments or locules. After filling the tubes with the stationary liquid, the tubes are inclined to a 30° angle from horizontal. In the ascending mode, the lighter mobile phase is applied to the bottom of the first tube by a constant flow pump, displacing the stationary phase as its volume attains the level of the hole in the disk. The mobile phase passes through this hole and enters into the next compartment, where the process continues until the mobile phase emerges from the uppermost locule. Finally, the two phases fill approximately half of each compartment. The dissolved essential oil subsequently introduced is subjected to a multistage partitioning process that leads to separation of the individual components. Whereas gravity contributes to the phase separation, rotation of the column assembly

(60–80 rpm) produces circular stirring of the two liquids to promote partition. If the descending mode is selected for separation, the heavier mobile phase is applied at the top of each column by switching a valve. An overview on applications of RLCC in natural products isolation inclusive of a detailed description of the device and the selection of appropriate solvent systems has been presented by Snyder et al. (1984).

Comparing RLCC to the aforementioned DCCC, one can particularly stress the superior flexibility of RLCC. While DCCC requires under all circumstances a two-phase system able to form droplets in the stationary phase, the choice of solvent systems with RLCC is nearly free. So the limitations of DCCC, when analyzing lipophilic samples, do not apply to RLCC. The separation of a mixture of terpenes has been presented by Kubeczka (1985). A different method, the high-speed centrifugal CCC developed by Ito and coworkers in the mid-1960s (Ito et al., 1966), has been applied to separate a variety of nonvolatile natural compounds; however, separation of volatiles has, strange to say, until now not seriously been evaluated.

2.3.3 Hyphenated Techniques

2.3.3.1 Gas Chromatography-Mass Spectrometry

The advantage of online coupling of a chromatographic device to a spectrometer is that complex mixtures can be analyzed in detail by spectral interpretation of the separated individual components. The coupling of a gas chromatograph with a mass spectrometer is the most often used and a well-established technique for the analysis of essential oils, due to the development of easy-to-handle powerful systems concerning sensitivity, data acquisition and processing, and above all their relatively low cost. The very first application of a GC-MS coupling for the identification of essential oil constituents using a capillary column was already published by Buttery et al. (1963). In those times, mass spectra have been traced on UV recording paper with a five-element galvanometer, and their evaluation was a considerable cumbersome task.

This has changed after the introduction of computerized mass digitizers yielding the mass numbers and the relative mass intensities. The different kinds of GC-MS couplings available at the end of the seventies of the last century have been described in detail by ten Noever de Brauw (1979). In addition, different types of mass spectrometers have been applied in GC-MS investigations such as magnetic sector instruments, quadrupole mass spectrometers, ion-trap analyzers (e.g., ion-trap detector), and time-of-flight mass spectrometers, which are the fastest MS analyzers and therefore used for very fast GC-MS systems (e.g., in comprehensive multidimensional GC-MS). Surprisingly, a time-of-flight mass spectrometer was used in the very first description of a GC-MS investigation of an essential oil mentioned before. From the listed spectrometers, the magnetic sector and quadrupole instruments can also be used for selective ion monitoring, to improve sensitivity for the analysis of target compounds and for discrimination of overlapping GC peaks.

The great majority of today's GC-MS applications utilize 1D capillary GC with quadrupole MS detection and electron ionization. Nevertheless, there are substantial numbers of applications using different types of mass spectrometers and ionization techniques. The proliferation of GC-MS applications is also a result of commercially available easy-to-handle dedicated mass spectral libraries (e.g., NIST/EPA/NIH 2005; WILEY Registry 2006; MassFinder 2007; and diverse printed versions such as Jennings and Shibamoto, 1980; Joulain and König, 1998; Adams, 1989, 1995, 2007 inclusive of retention indices) providing identification of the separated compounds. However, this type of identification has the potential of producing some unreliable results, if no additional information is used, since some compounds, for example, the sesquiterpene hydrocarbons α-cuprenene and β-himachalene, exhibit identical fragmentation pattern and only very small differences of their retention index values. This example demonstrates impressively that even a good library match and the additional use of retention data may lead in some cases to questionable results, and therefore require additional analytical data, for example, from NMR measurements.

2.3.3.1.1 GC-Chemical Ionization-MS and GC-Tandem MS

Although GC-electron impact (EI)-MS is a very useful tool for the analysis of essential oils, this technique can sometimes be not selective enough and requires more sophisticated techniques such as GC-chemical ionization-MS (GC-CI-MS) and GC-tandem MS (GC-MS-MS). The application of CI-MS using different reactant gases is particularly useful, since many terpene alcohols and esters fail to show a molecular ion. The use of OH^- as a reactant ion in negative CI-MS appeared to be an ideal solution to this problem. This technique yielded highly stable quasi-molecular ions M–H, which are often the only ions in the obtained spectra of the aforementioned compounds. As an example, the EI and CI spectra of isobornyl isovalerate—a constituent of valerian oil—shall be quoted (Bos et al., 1982). The respective EI mass spectrum shows only a very small molecular ion at 238. Therefore, the chemical ionization spectra of isobornyl acetate were performed with isobutene as a reactant gas a $[C_{10}H_{17}]^+$ cation and in the negative CI mode with OH^- as a reactant gas two signals with the masses 101, the isovalerate anion, and 237 the quasi-molecular ion $[M–H]^-$. Considering all these obtained data, the correct structure of the oil constituent could be deduced. The application of isobutane and ammonia as reactant gases has been presented by Schultze et al. (1992), who investigated sesquiterpene hydrocarbons by GC-CI-MS. Fundamental aspects of chemical ionization MS have been reviewed by Bruins (1987), discussing the different reactant gases applied in positive and negative ion chemical ionization and their applications in essential oil analysis.

The utilization of GC-MS-MS to the analysis of a complex mixture will be shown in Figure 2.6. In the investigated vetiver oil (Cazaussus et al., 1988), one constituent, the norsesquiterpene ketone khusimone, has been identified by using GC-MS-MS in the collision-activated dissociation mode. The molecular ion at *m/z* 204 exhibited a lot of daughter ions, but only one of them gave a daughter ion at *m/z* 108, a fragment rarely occurring in sesquiterpene derivatives so that the presence of khusimone could be undoubtedly identified.

2.3.3.2 High-Resolution GC-FTIR Spectroscopy

A further hyphenated technique, providing valuable analytical information, is the online coupling of a gas chromatograph with a FTIR spectrometer. The capability of IR spectroscopy to provide discrimination between isomers makes the coupling of a gas chromatograph to an FTIR spectrometer

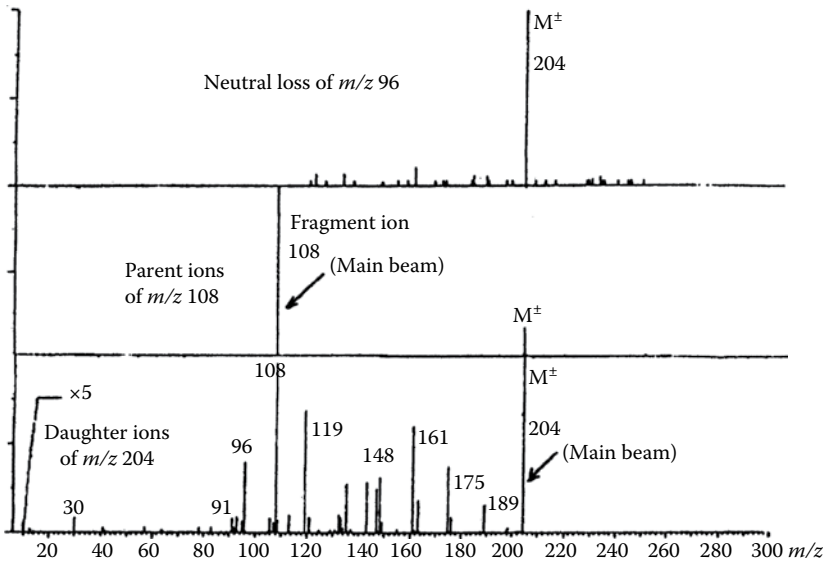

FIGURE 2.6 GC-EIMS-MS of khusimone of vetiver oil. (From Cazaussus, A. et al., *Chromatographia*, 25, 865, 1988. With permission.)

suited as a complementary method to GC/MS for the analysis of complex mixtures like essential oils. The GC/FTIR device consists basically of a capillary gas chromatograph and an FTIR spectrometer including a dedicated computer and ancillary equipment. As each GC peak elutes from the GC column, it enters a heated IR measuring cell, the so-called light pipe, usually a gold-plated glass tube with IR transparent windows. There, the spectrum is measured as an interferogram from which the familiar absorbance spectrum can be calculated by computerized Fourier transformation. After passing the light pipe, the effluent is directed back into the FID of the gas chromatograph. More detailed information on the experimental setup was given by Herres et al. (1986) and Herres (1987).

In the latter publication, for example, the vapor-phase IR spectra of all the four isomers of pulegol and dihydrocarveol are shown, which have been extracted from a GC/FTIR run. These examples convincingly demonstrate the capability of distinguishing geometrical isomers with the aid of vapor-phase IR spectra, which cannot be achieved by their mass spectra. A broad application of GC-FTIR in the analysis of essential oils, however, is limited by the lack of sufficient vapor-phase spectra of uncommon compounds, which are needed for reference use, since the spectra of isolated molecules in the vapor phase can be significantly different from the corresponding condensed-phase spectra.

A different approach has been published by Reedy et al. in 1985, using a cryogenically freezing of the GC effluent admixed with an inert gas (usually argon) onto a rotating disk maintained at liquid He temperature to form a solid matrix trace. After the separation, reflection absorption spectra can be obtained from the deposited solid trace. A further technique published by Bourne et al. (1990) is the subambient trapping, whereby the GC effluent is cryogenically frozen onto a moving IR transparent window of zinc selenide (ZnSe). An advantage of the latter technique is that the unlike larger libraries of conventional IR spectra can be searched in contrast to the limited number of vapor-phase spectra and those obtained by matrix isolation. A further advantage of both cryogenic techniques is the significant higher sensitivity, which exceeds the detection limits of a light pipe instrument by approximately two orders of magnitude.

Comparing GC/FTIR and GC/MS, advantages and limitations of each technique become visible. The strength of IR lies—as discussed before—in distinguishing isomers, whereas identification of homologues can only be performed successfully by MS. The logical and most sophisticated way to overcome these limitations has been the development of a combined GC/FTIR/MS instrument, whereby simultaneously IR and mass spectra can be obtained.

2.3.3.3 GC-UV Spectroscopy

The instrumental coupling of gas chromatograph with a rapid scanning UV spectrometer has been presented by Kubeczka et al. (1989). In this study, a UV-VIS diode-array spectrometer (Zeiss, Oberkochen, FRG) with an array of 512 diodes was used, which provided continuous monitoring in the range of 200–620 nm. By interfacing the spectrometer via fiber optics to a heated flow cell, which was connected by short heated capillaries to the GC column effluent, interferences of chromatographic resolution could be minimized. With the aid of this device, several terpene hydrocarbons have been investigated. In addition to displaying individual UV spectra, the available software rendered the analyst to define and to display individual window traces, 3D plots, and contour plots, which are valuable tools for discovering and deconvoluting gas chromatographic unresolved peaks.

2.3.3.4 Gas Chromatography-Atomic Emission Spectroscopy

A device for the coupling of capillary GC with atomic emission spectroscopy (GC-AES) has been presented by Wylie and Quimby (1989). By means of this coupling, 23 elements of a compound including all elements of organic substances separated by GC could be selectively detected providing the analyst not only with valuable information on the elemental composition of the individual components of a mixture but also with the percentages of the elemental composition. The device incorporates a microwave-induced helium plasma at the outlet of the column coupled to an optical emission spectrometer. From the 15 most commonly occurring elements in organic compounds,

up to 8 could be detected and measured simultaneously, for example, C, O, N, and S, which are of importance with respect to the analysis of essential oils. The examples given in the literature (e.g., Wylie and Quimby, 1989; Bicchi et al., 1992; David and Sandra, 1992; Jirovetz et al., 1992; Schultze, 1993) indicate that the GC-AES coupling can provide the analyst with additional valuable information, which are to some extent complementary to the date obtained by GC-MS and GC-FTIR, making the respective library searches more reliable and more certain.

However, the combined techniques GC-UV and GC-AES have not gained much importance in the field of essential oil research, since UV spectra offer only low information and the coupling of a GC-AES, yielding the exact elemental composition of a component, can to some extent be obtained by precise mass measurement. Nevertheless, the online coupling GC-AES is still today efficiently used in environmental investigations.

2.3.3.5 Gas Chromatography-Isotope Ratio Mass Spectrometry

In addition to enantioselective capillary GC, the online coupling of GC with isotope-ratio MS (GC-IRMS) is an important technique in authentication of food flavors and essential oil constituents. The online combustion of effluents from capillary gas chromatographic separations to determine the isotopic compositions of individual components from complex mixtures was demonstrated by Matthews and Hayes (1978). On the basis of this work, the online interfacing of capillary GC with IRMS was later improved. With the commercially available GC-combustion IRMS device, measurements of the ratios of the stable isotopes $^{13}C/^{12}C$ have been accessible and respective investigations have been reported in several papers (e.g., Bernreuther et al., 1990; Carle et al., 1990; Braunsdorf et al., 1992, 1993; Frank et al., 1995; Mosandl and Juchelka, 1997). A further improvement was the development of the GC-pyrolysis-IRMS (GC-P-IRMS) making measurements of $^{18}O/^{16}O$ ratios and later $^{2}H/^{1}H$ ratios feasible (Juchelka et al., 1998; Ruff et al., 2000; Hör et al., 2001; Mosandl, 2004). Thus, the GC-P-IRMS device (Figure 2.7) appears today as one of the most sophisticated instruments for the appraisal of the genuineness of natural mixtures.

2.3.3.6 High-Performance Liquid Chromatography-Gas Chromatography

The online coupling of an HPLC device to a capillary gas chromatograph offers a number of advantages, above all higher column chromatographic efficiency, simple and rapid method development, simple cleanup of samples from complex matrices, and effective enrichment of the

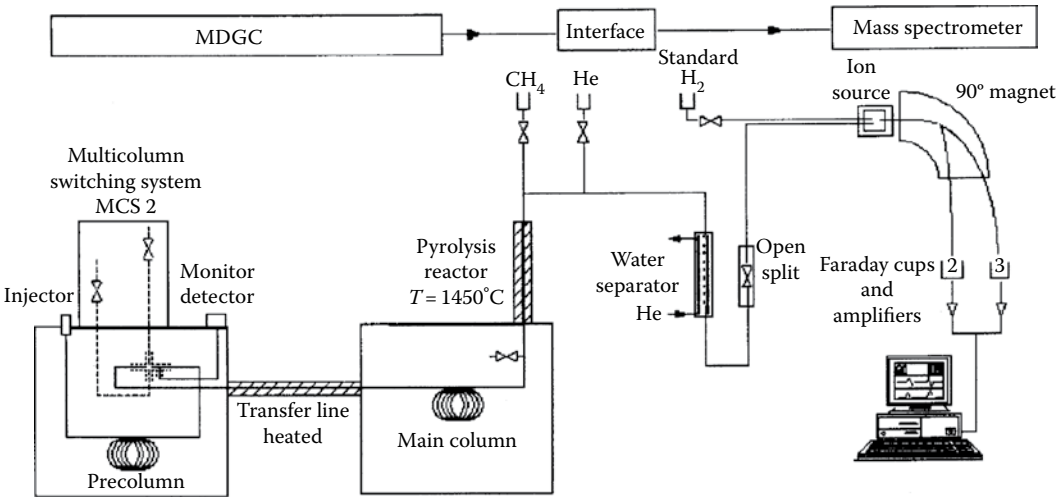

FIGURE 2.7 Scheme of an MDGC-C/P-IRMS device. (From Sewenig, S. et al., *J. Agric. Food Chem.*, 53, 838, 2005. With permission.)

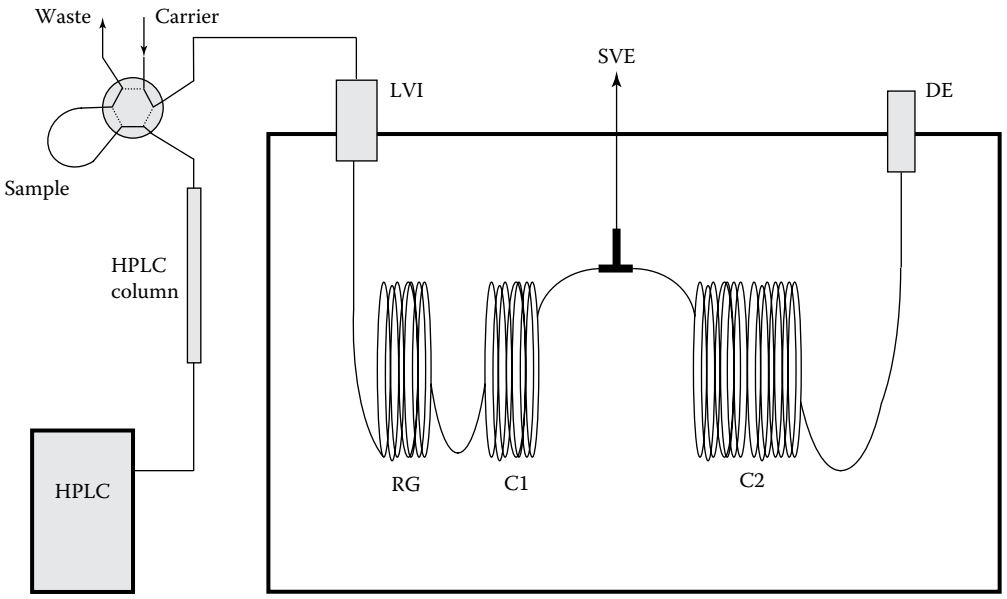

FIGURE 2.8 Basic arrangement of an HPLC-GC device with a sample loop interface. RG, retention gap; C1, retaining column; C2, analytical column; LVI, large volume injector; and SVE, solvent vapor exit.

components of interest; additionally, the entire analytical procedure can easily be automated, thus increasing accuracy and reproducibility. The commercially available HPLC-GC coupling consists of an HPLC device that is connected with a capillary gas chromatograph via an interface allowing the transfer of HPLC fractions. Two different types of interfaces have been often used. The on-column interface is a modification of the on-column injector for GC; it is particularly suited for the transfer of fairly small fraction containing volatile constituents (Dugo et al., 1994; Mondello et al., 1994a,b, 1995). The second interface uses a sample loop and allows to transfer large sample volumes (up to 1 mL) containing components with limited volatilities. Figure 2.8 gives a schematic view of such an LC-GC instrument. In the shown position of the six-port valve, the desired fraction of the HPLC effluent is stored in the sample loop, while the carrier gas is passed through the GC columns. After switching the valve, the content of the sample loop is driven by the carrier gas into the large volume injector and vaporized and enters the precolumns, where the sample components are retained and most of the solvent vapor can be removed through the solvent vapor exit. After closing this valve and increasing the GC-oven temperature, the sample components are volatilized and separated in the main column reaching the detector. The main drawback of this technique, however, may be the loss of highly volatile compounds that are vented together with the solvent. As an example of an HPLC-GC investigation, the preseparation of lemon oil with gradient elution into four fractions is quoted (Munari et al., 1990). The respective gas chromatograms of the individual fractions exhibit good separation into hydrocarbons, esters, carbonyls, and alcohols, facilitating gas chromatographic separation and identification. Due to automation of all analytical steps involved, the manual operations are significantly reduced, and very good reproducibility was obtained. In three excellent review articles, the different kinds of HPLC-GC couplings are discussed in detail, describing their advantages and limitations with numerous references cited therein (Mondello et al., 1996, 1999; Dugo et al., 2003).

2.3.3.7 HPLC-MS, HPLC-NMR Spectroscopy

The online couplings of HPLC with MS and NMR spectroscopy are further important techniques combining high-performance separation with structurally informative spectroscopic techniques,

but they are mainly applied to nonvolatile mixtures and shall not be discussed in more detail here, although they are very useful for investigating plant extracts.

Some details concerning the different ionization techniques used in HPLC-MS have been presented among other things by Dugo et al. (2005).

2.3.3.8 Supercritical Fluid Extraction–Gas Chromatography

Although SFE is not a chromatographic technique, separation of mixtures can be obtained during the extraction process by varying the physical properties such as temperature and pressure to obtain fractions of different composition. Detailed reviews on the physical background of SFE and its application to natural products analysis inclusive of numerous applications have been published by Modey et al. (1995) and more recently by Pourmortazavi and Hajimirsadeghi (2007). The different types of couplings (offline and online) have been presented by several authors. Houben et al. (1990) described an online coupling of SFE with capillary GC using a programmed temperature vaporizer as an interface. Similar approaches have been used by Blanch et al. (1994) in their investigations of rosemary leaves and by Ibanez et al. (1997) studying Spanish raspberries. In both the last two papers, an offline procedure was applied. A different device has been used by Hartonen et al. (1992) in a study of the essential oil of *Thymus vulgaris* using a cooled stainless steel capillary for trapping the volatiles connected via a six-port valve to the extraction vessel and the GC column. After sampling of the volatiles within the trap, they have been quickly vaporized and flushed into the GC column by switching the valve. The recoveries of thyme components by SFE-GC were compared with those obtained from hydrodistilled thyme oil by GC exhibiting a good agreement. The SFE-GC analyses of several flavor and fragrance compounds of natural products by transferring the extracted compounds from a small SFE cell directly into a GC capillary has already been presented by Hawthorne et al. (1988). By inserting the extraction cell outlet restrictor (a 20 μm ID capillary) into the GC column through a standard on-column injection port, the volatiles were transferred and focused within the column at 40°C, followed by rapid heating to 70°C (30°C/min) and successive usual temperature programming. The suitability of that approach has been demonstrated with a variety of samples including rosemary, thyme, cinnamon, spruce needles, orange peel, and cedar wood. In a review article from Greibrokk, published in 1995, numerous applications of SFE connected online with GC and other techniques, the different instruments, and interfaces have been discussed, including the main parameters responsible for the quality of the obtained analytical results. In addition, the instrumental setups for SFE-LC and SFE-SFC couplings are given.

2.3.3.9 Supercritical Fluid Chromatography-Gas Chromatography

Online coupling of SFC with GC has sporadically been used for the investigation of volatiles from aromatic herbs and spices. The requirements for instrumentation regarding the pumps, the restrictors, and the detectors are similar to those of SFE-GC. Additional parts of the device are the separation column and the injector, to introduce the sample into the mobile phase and successively into the column. The most common injector type in SFC is the high-pressure valve injector, similar to those used in HPLC. With this valve, the sample is loaded at ambient pressure into a sample loop of defined size and can be swept into the column after switching the valve to the injection position. The separation columns used in SFC may be either packed or open tubular columns with their respective advantages and disadvantages. The latter mentioned open tubular columns for SFC can be compared with the respective GC columns; however, they must have smaller internal diameter. With regard to the detectors used in SFC, the FID is the most common applied detector, presuming that no organic modifiers have been admixed to the mobile phase. In that case, for example, a UV detector with a high-pressure flow cell has to be taken into consideration.

In a paper presented by Yamauchi and Saito (1990), cold-pressed lemon-peel oil has been separated by semipreparative SFC into three fractions (hydrocarbons, aldehydes and alcohols), and esters together with other oil constituents. The obtained fractions were afterward analyzed by capillary GC. SFC has also often been combined with SFE prior to chromatographic separation in

plant volatile oil analysis, since in both techniques the same solvents are used, facilitating an online coupling. SFE and online-coupled SFC have been applied to the analysis of turmeric, the rhizomes of *Curcuma longa* L., using modified carbon dioxide as the extractant, yielding fractionation of turmerones curcuminoids in a single run (Sanagi et al., 1993). A multidimensional SFC-GC system was developed by Yarita et al. (1994) to separate online the constituents of citrus essential oils by stepwise pressure programming. The eluting fractions were introduced into a split/splitless injector of a gas chromatograph and analyzed after cryofocusing prior to GC separation. An SFC-GC investigation of cloudberry seed oil extracted with supercritical carbon dioxide was described by Manninen and Kallio (1997), in which SFC was mainly used for the separation of the volatile constituents from the low-boiling compounds, such as triacylglycerols. The volatiles were collected in a trap column and refocused before being separated by GC. Finally, an online technique shall be mentioned by which the compounds eluting from the SFC column can be completely transferred to GC, but also for selective or multistep heart-cutting of various sample peaks as they elute from the SFC column (Levy et al., 2005).

2.3.3.10 Couplings of SFC-MS and SFC-FTIR Spectroscopy

Both coupling techniques such as SFC-MS and SFC-FTIR have nearly exclusively been used for the investigation of low-volatile more polar compounds. Arpino published in 1990 a comprehensive article on the different coupling techniques in SFC-MS, which have been presented up to 1990 including 247 references. A short overview of applications using SFC combined with benchtop mass spectrometers was published by Ramsey and Raynor (1996). However, the only paper concerning the application of SFC-MS in essential oil research was published by Blum et al. (1997). With the aid of a newly developed interface and an injection technique using a retention gap, investigations of thyme extracts have been successfully performed.

The application of SFC-FTIR spectroscopy for the analysis of volatile compounds has also rarely been reported. One publication found in the literature refers to the characterization of varietal differences in essential oil components of hops (Auerbach et al., 2000). In that paper, the IR spectra of the main constituents were taken as films deposited on AgCl disks and compared with spectra obtained after chromatographic separation in a flow cell with IR transparent windows, exhibiting a good correlation.

2.3.4 Identification of Multicomponent Samples without Previous Separation

In addition to chromatographic separation techniques including hyphenated techniques, several spectroscopic techniques have been applied to investigate the composition of essential oils without previous separation.

2.3.4.1 UV Spectroscopy

UV spectroscopy has only little significance for the direct analysis of essential oils due to the inability to provide uniform information on individual oil components. However, for testing the presence of furanocoumarins in various citrus oils, which can cause photodermatosis when applied externally, UV spectroscopy is the method of choice. The presence of those components can be easily determined due to their characteristic UV absorption. In the European pharmacopoeia, for example, quality assessment of lemon oil, which has to be produced by cold pressing, is therefore performed by UV spectroscopy in order to exclude cheaper distilled oils.

2.3.4.2 IR Spectroscopy

Several attempts have also been made to obtain information about the composition of essential oils using IR spectroscopy. One of the first comprehensive investigations of essential oils was published by Bellanato and Hidalgo (1971) in the book *Infrared Analysis of Essential Oils* in which the IR spectra of approximately 200 essential oils and additionally of more than 50 pure

reference components have been presented. However, the main disadvantage of this method is the low sensitivity and selectivity of the method in the case of mixtures with a large number of components and, second, the unsolvable problem when attempting to quantitatively measure individual component concentrations.

New approaches to analyze essential oils by vibrational spectroscopy using attenuated reflection (ATR) IR spectroscopy and NIR-FT-Raman spectroscopy have recently been published by Baranska et al. (2005) and numerous papers cited therein. The main components of an essential oil can be identified by both spectroscopic techniques using the spectra of pure oil constituents as references. The spectroscopic analysis is based on characteristic key bands of the individual constituents and made it, for example, possible to discriminate the oil profiles of several eucalyptus species. As can be taken from this paper, valuable information can be obtained as a result of the combined application of ATR-IR and NIR-FT-Raman spectroscopy. Based on reference GC measurements, valuable calibration equations have been developed for numerous essential oil plants and related essential oils in order to quantify the amount of individual oil constituents applying different suitable chemometric algorithms. Main advantages of those techniques are their ability to control the quality of essential oils very fast and easily and, above all, their ability to quantify and analyze the main constituents of essential oils *in situ*, that means in living plant tissues without any isolation process, since both techniques are not destructive.

2.3.4.3 Mass Spectrometry

MS and proton NMR spectroscopy have mainly been used for structure elucidation of isolated compounds. However, there are some reports on mass spectrometric analyses of essential oils. One example has been presented by Grützmacher (1982). The depicted mass spectrum (Figure 2.9) of an essential oil exhibits some characteristic molecular ions of terpenoids with masses at *m/z* 136, 148, 152, and 154. By the application of a double focusing mass spectrometer and special techniques analyzing the decay products of metastable ions, the components anethole, fenchone, borneol, and cineole could be identified, while the assignment of the mass 136 proved to be problematic.

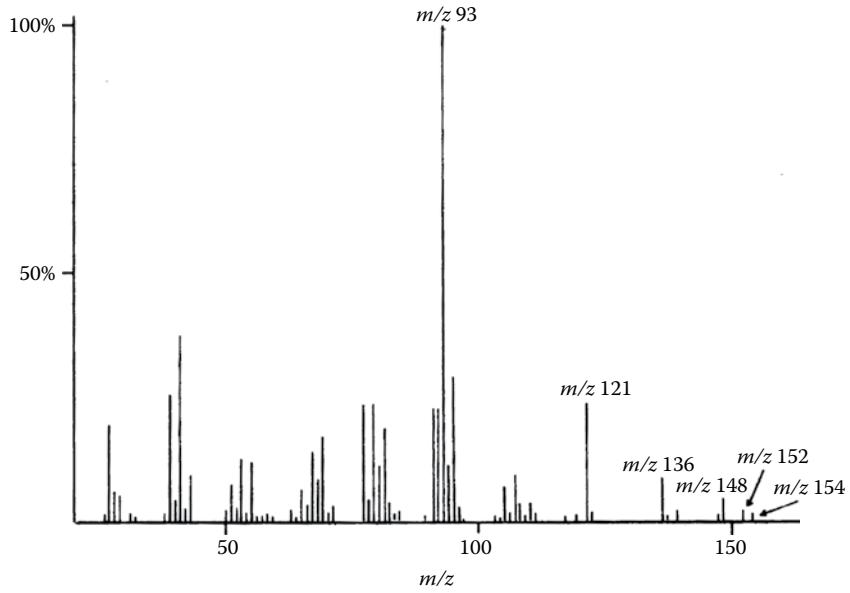

FIGURE 2.9 EI-mass spectrum of an essential oil. (From Grützmacher, H.F., 1982. In Ätherische Öle: Analytik, Physiologie, Zusammensetzung K.H. Kubeczka (ed.), pp. 1–24. Stuttgart, Germany: Georg Thieme Verlag. With permission.)

A different approach has been used by Schultze et al. (1986), investigating secondary metabolites in dried plant material by direct mass spectrometric measurement. The small samples (0.1–2 mg, depending on the kind of plant drug) were directly introduced into a mass spectrometer by means of a heatable direct probe. By heating the solid sample, stored in a small glass crucible, various substances are released depending on the applied temperature, and subsequently their mass spectra can be taken. With the aid of this technique, numerous medicinal plant drugs have been investigated and their main vaporizable components could be identified.

2.3.4.4 ^{13}C-NMR Spectroscopy

^{13}C-NMR spectroscopy is generally used for the elucidation of molecular structures of isolated chemical species. The application of ^{13}C-NMR spectroscopy to the investigation of complex mixtures is relatively rare. However, the application of ^{13}C-NMR spectroscopy to the analysis of essential oils and similar complex mixtures offers particular advantages, as have been shown in the past (Formaček and Kubeczka, 1979, 1982a; Kubeczka, 2002), to confirm analytical results obtained by GC-MS and for solving certain problems encountered with nonvolatile mixture components or thermally unstable compounds, since analysis is performed at ambient temperature.

The qualitative analysis of an essential oil is based on comparison of the oil spectrum, using broadband decoupling, with spectra of pure oil constituents, which should be recorded under identical conditions regarding solvent, temperature, and so on to ensure that differences in the chemical shifts for individual ^{13}C-NMR lines of the mixture and of the reference substance are negligible. As an example, the identification of the main constituent of celery oil is shown (Figure 2.10). This constituent can be easily identified as limonene by the corresponding reference spectrum. Minor constituents give rise to less intensive signals that can be recognized after a vertical expansion of the spectrum. For recognition of those signals, also a horizontal expansion of the spectrum is advantageous.

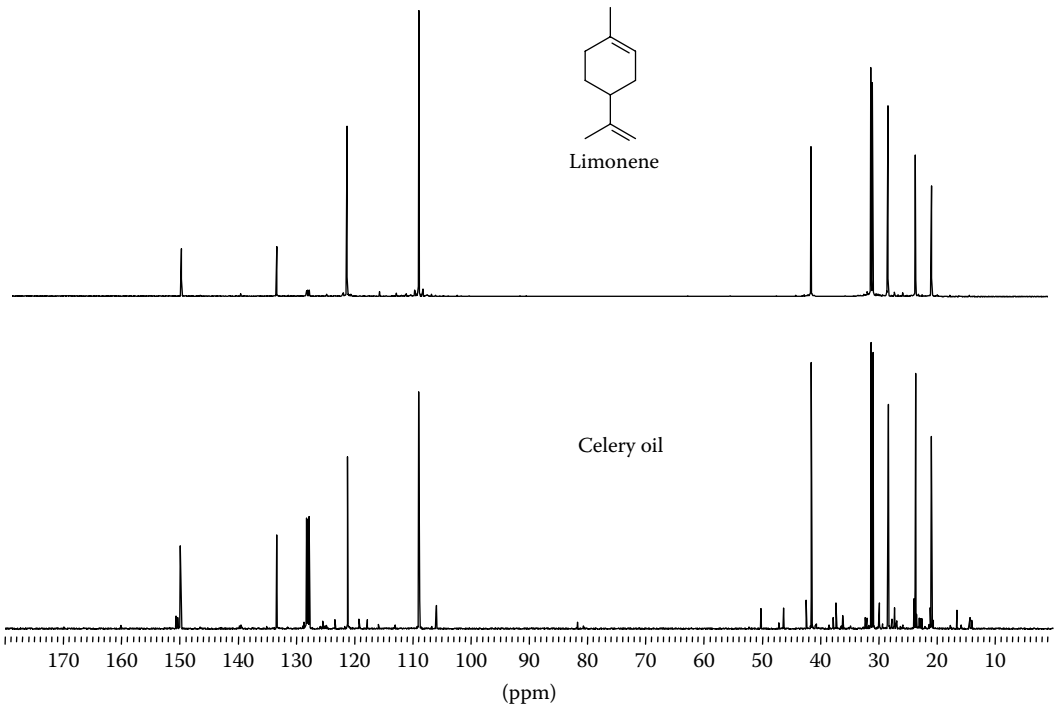

FIGURE 2.10 Identification of limonene in celery oil by ^{13}C-NMR spectroscopy.

The sensitivity of the ^{13}C-NMR technique is limited by diverse factors such as rotational sidebands, ^{13}C–^{13}C couplings, and so on, and at least by the accumulation time. For practical use, the concentration of 0.1% of a component in the entire mixture has to be seen as an interpretable limit. A very pretentious investigation has been presented by Kubeczka (1989). In the investigated essential oil, consisting of more than 80 constituents, approximately 1200 signals were counted after a horizontal and vertical expansion in the obtained broadband decoupled ^{13}C-NMR spectrum, which reflects impressively the complex composition of that oil. However, the analysis of such a complex mixture is made difficult by the immense density of individual lines, especially in the aliphatic region of the spectrum, making the assignments of lines to individual components ambiguous. Besides, qualitative analysis quantification of the individual sample components is accessible as described by Formaček and Kubeczka (1982b). After elimination of the ^{13}C-NMR signals of nonprotonated nuclei and calculation of average signal intensity per carbon atom as a measurement characteristic, it has been possible to obtain satisfactory results as shown by comparison with gas chromatographic analyses.

During the last years, a number of articles have been published by Casanova and coworkers (e.g., Bradesi et al. (1996) and references cited therein). In addition, papers dealing with computer-aided identification of individual components of essential oils after ^{13}C-NMR measurements (e.g., Tomi et al., 1995), and investigations of chiral oil constituents by means of a chiral lanthanide shift reagent by ^{13}C-NMR spectroscopy have been published (Ristorcelli et al., 1997).

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3 Sources of Essential Oils

Chlodwig Franz and Johannes Novak

CONTENTS

3.1 "ESSENTIAL OIL–BEARING PLANTS": ATTEMPT OF A DEFINITION

Essential oils are complex mixtures of volatile compounds produced by living organisms and isolated by physical means only (pressing and distillation) from a whole plant or plant part of known taxonomic origin. The respective main compounds are mainly derived from three biosynthetic pathways only, the mevalonate pathway leading to sesquiterpenes, the methyl-erythritol pathway leading to mono- and diterpenes, and the shikimic acid pathway *en route* to phenylpropenes. Nevertheless, there are an almost uncountable number of single substances and a tremendous variation in the composition of essential oils. Many of these volatile substances have diverse ecological functions. They can act as internal messengers, as defensive substances against herbivores, or as volatiles not only directing natural enemies to these herbivores but also attracting pollinating insects to their host (Harrewijn et al., 2001).

All plants possess principally the ability to produce volatile compounds, quite often, however, only in traces. "Essential oil plants" in particular are those plant species delivering an essential oil

of commercial interest. Two principal circumstances determine a plant to be used as an essential oil plant:

1. A unique blend of volatiles like the flower scents in rose (*Rosa* spp.), jasmine (*Jasminum sambac*), or tuberose (*Polianthes tuberosa*). Such flowers produce and immediately emit the volatiles by the epidermal layers of their petals (Bergougnoux et al., 2007). Therefore, the yield is even in intensive smelling flowers very low, and besides distillation special techniques, as an example, enfleurage has to be applied to recover the volatile fragrance compounds.
2. Secretion and accumulation of volatiles in specialized anatomical structures. These lead to higher concentrations of the essential oil in the plant. Such anatomical storage structures for essential oils can be secretory idioblasts (secretory cells), cavities/ducts, or glandular trichomes (Fahn, 1979, 1988; colorfully documented by Svoboda et al, 2000).

Secretory idioblasts are individual cells producing an essential oil in large quantities and retaining the oil within the cell like the essential oil idioblasts in the roots of *Vetiveria zizanioides* that occurs within the cortical layer and close to the endodermis (Bertea and Camusso, 2002). Similar structures containing essential oils are also formed in many flowers, for example, *Rosa* sp., *Viola* sp., or *Jasminum* sp.

Cavities or ducts consist of extracellular storage space that originate either by schizogeny (created by the dissolution of the middle lamella between the duct initials and formation of an intercellular space) or by lysogeny (programmed death and dissolution of cells). In both cases, the peripheral cells are becoming epithelial cells highly active in synthesis and secretion of their products into the extracellular cavities (Pickard, 2008). Schizogenic oil ducts are characteristic for the Apiaceae family, for example, *Carum carvi*, *Foeniculum vulgare*, or *Cuminum cyminum*, but also for the Hypericaceae or Pinaceae family. Lysogenic cavities are found in Rutaceae (*Citrus* sp., *Ruta graveolens*), Myrtaceae (e.g., *Syzygium aromaticum*), and others.

Secreting trichomes (glandular trichomes) can be divided into two main categories: peltate and capitate trichomes. Peltate glands consist of a basal epidermal cell, a neck–stalk cell, and a secreting head of 4–16 cells with a large subcuticular space on the apex in which the secretion product is accumulated. The capitate trichomes possess only 1–4 secreting cells with only a small subcuticular space (Werker, 1993; Maleci Bini and Giuliani, 2006). Such structures are typical for Lamiaceae (the mint family), but also for *Pelargonium* sp.

The monoterpene biosynthesis in different species of Lamiaceae, for example, sage (*Salvia officinalis*) and peppermint (*Mentha piperita*), is restricted to a brief period early in leaf development (Croteau et al., 1981; Gershenzon et al., 2000). The monoterpene biosynthesis in peppermint reaches a maximum in 15-day-old leaves; only very low rates were observed in leaves younger than 12 days or older than 20 days. The monoterpene content of the peppermint leaves increased rapidly up to day 21, then leveled off, and kept stable for the remainder of the leaf life (Gershenzon et al., 2000).

The composition of the essential oil often changes between different plant parts. Phytochemical polymorphism is often the case between different plant organs. In *Origanum vulgare* ssp. *hirtum*, a polymorphism within a plant could even be detected on a much lower level, between different oil glands of a leaf (Johnson et al., 2004). This form of polymorphism seems to be not frequently occurring; differences in the composition between oil glands are more often related to the age of the oil glands (Grassi et al., 2004; Johnson et al., 2004; Novak et al., 2006a; Schmiderer et al., 2008).

Such polymorphisms can also be found quite frequently when comparing the essential oil composition of individual plants of a distinct species (intraspecific variation, “chemotypes”) and is based on the plants” genetic background.

The differences in the complex composition of two essential oils of one kind may sometimes be difficult to assign to specific chemotypes or to differences arising in the consequence of the reactions of the plants to specific environmental conditions, for example, to different growing

locations. In general, the differences due to genetic differences are much bigger than by different environmental conditions. However, many intraspecific polymorphisms are probably not yet detected or have been described only recently even for widely used essential oil crops like sage (Novak et al., 2006b).

3.2 PHYTOCHEMICAL VARIATION

3.2.1 Chemotaxonomy

The ability to accumulate essential oils is not omnipresent in plants but scattered throughout the plant kingdom, in many cases, however, very frequent within—or a typical character of—certain plant families. From the taxonomical and systematic point of view, not the production of essential oils is the distinctive feature since this is a quite heterogeneous group of substances, but either the type of secretory containers (trichomes, oil glands, lysogenic cavities, or schizogenic oil ducts) or the biosynthetically specific group of substances, for example, mono- or sesquiterpenes and phenylpropenes; the more a substance is deduced in the biosynthetic pathway, the more specific it is for certain taxa: monoterpenes are typical for the genus *Mentha*, but menthol is characteristic for *M. piperita* and *Mentha arvensis* ssp. *piperascens* only; sesquiterpenes are common in the *Achillea–millefolium* complex, but only *Achillea roseoalba* (2×) and *Achillea collina* (4×) are able to produce matricine as precursor of (the artifact) chamazulene (Vetter et al., 1997). On the other hand, the phenylpropanoid eugenol, typical for cloves (*S. aromaticum*, Myrtaceae), can also be found in large amounts in distant species, for example, cinnamon (*Cinnamomum zeylanicum*, Lauraceae) or basil (*Ocimum basilicum*, Lamiaceae); as sources for anethole are known not only aniseed (*Pimpinella anisum*) and fennel (*F. vulgare*), which are both Apiaceae, but also star anise (*Illicium verum*, Illiciaceae), *Clausena anisata* (Rutaceae), *Croton zehntneri* (Euphorbiaceae), or *Tagetes lucida* (Asteraceae). Finally, eucalyptol (1,8-cineole)—named after its occurrence in *Eucalyptus* sp. (Myrtaceae)—may also be a main compound of the essential oil of galangal (*Alpinia officinarum*, Zingiberaceae), bay laurel (*Laurus nobilis*, Lauraceae), Japan pepper (*Zanthoxylum piperitum*, Rutaceae), and a number of plants of the mint family, for example, sage (*S. officinalis*, *Salvia fruticosa*, *Salvia lavandulifolia*), rosemary (*Rosmarinus officinalis*), and mints (*Mentha* sp.). Taking the aforementioned facts into consideration, chemotaxonomically relevant are (therefore) common or distinct pathways, typical fingerprints, and either main compounds or very specific even minor or trace substances (e.g., δ-3-carene to separate *Citrus grandis* from other *Citrus* sp. [Gonzalez et al., 2002]).

The plant families comprising species that yield a majority of the most economically important essential oils are not restricted to one specialized taxonomic group but are distributed among all plant classes: gymnosperms, for example, the families Cupressaceae (cedarwood, cedar leaf, juniper oil, etc.) and Pinaceae (pine and fir oils), and angiosperms, and among them within Magnoliopsida, Rosopsida, and Liliopsida. The most important families of dicots are Apiaceae (e.g., fennel, coriander, and other aromatic seed/root oils), Asteraceae or Compositae (chamomile, wormwood, tarragon oil, a.s.o), Geraniaceae (geranium oil), Illiciaceae (star anise oil), Lamiaceae (mint, patchouli, lavender, oregano, and many other herb oils), Lauraceae (litsea, camphor, cinnamon, sassafras oil, etc.), Myristicaceae (nutmeg and mace), Myrtaceae (myrtle, cloves, and allspice), Oleaceae (jasmine oil), Rosaceae (rose oil), and Santalaceae (sandalwood oil). In monocots (Liliopsida), it is substantially restricted to Acoraceae (calamus), Poaceae (vetiver and aromatic grass oils), and Zingiberaceae (e.g., ginger and cardamom).

Apart from the phytochemical group of substances typical for a taxon, the chemical outfit depends, furthermore, on the specific genotype; the stage of plant development, also influenced by environmental factors; and the plant part (see Section 3.3.2.1). Considering all these influences, chemotaxonomic statements and conclusions have to be based on comparable material, grown and harvested under comparable circumstances.

3.2.2 Inter- and Intraspecific Variation

Knowledge on biochemical systematics and the inheritance of phytochemical characters depends on extensive investigations of taxa (particularly species) and populations on single-plant basis, respectively, and several examples of genera show that the taxa do indeed display different patterns.

3.2.2.1 Lamiaceae (Labiatae) and Verbenaceae

The presumably largest genus among the Lamiaceae is *sage* (*Salvia* L.) consisting of about 900 species widely distributed in the temperate, subtropical, and tropical regions all over the world with major centers of diversity in the Mediterranean, in Central Asia, the Altiplano from Mexico throughout Central and South America, and in southern Africa. Almost 400 species are used in traditional and modern medicine, as aromatic herbs or ornamentals worldwide; among them are *S. officinalis*, *S. fruticosa*, *Salvia sclarea*, *Salvia divinorum*, *Salvia miltiorrhiza*, and *Salvia pomifera*, to name a few. Many applications are based on nonvolatile compounds, for example, diterpenes and polyphenolic acids. Regarding the essential oil, there are a vast number of mono- and sesquiterpenes found in sage but, in contrast to, for example, *Ocimum* sp. and *Perilla* sp. (also Lamiaceae), no phenylpropenes were detected.

To understand species-specific differences within this genus, the Mediterranean *S. officinalis* complex (*S. officinalis*, *S. fruticosa*, and *S. lavandulifolia*) will be confronted with the *Salvia stenophylla* species complex (*S. stenophylla*, *Salvia repens*, and *Salvia runcinata*) indigenous to South Africa: in the *S. officinalis* group, usually α- and β-thujones, 1,8-cineole, camphor, and, in some cases, linalool, β-pinene, limonene, or *cis*-sabinyl acetate are the prevailing substances, whereas in the *S. stenophylla* complex, quite often sesquiterpenes, for example, caryophyllene or α-bisabolol, are main compounds.

Based on taxonomical studies of *Salvia* spp. (Hedge, 1992; Skoula et al., 2000; Reales et al., 2004) and a recent survey concerning the chemotaxonomy of *S. stenophylla* and its allies (Viljoen et al., 2006), Figure 3.1 shows the up-to-now-identified chemotypes within these taxa. Comparing the data

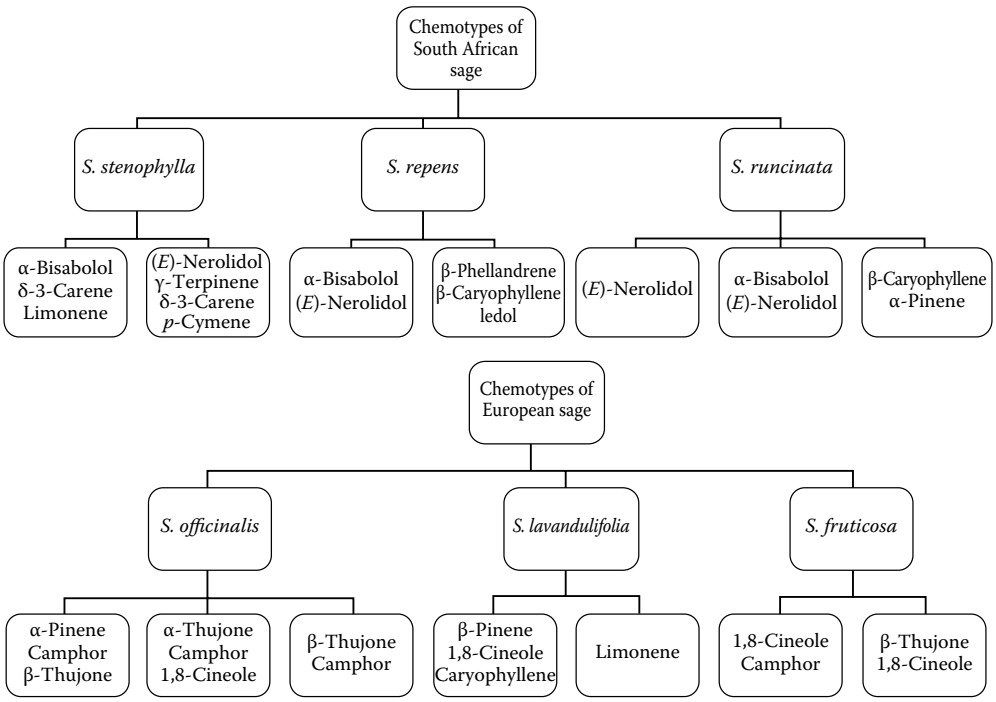

FIGURE 3.1 Chemotypes of some South African and European *Salvia* species.

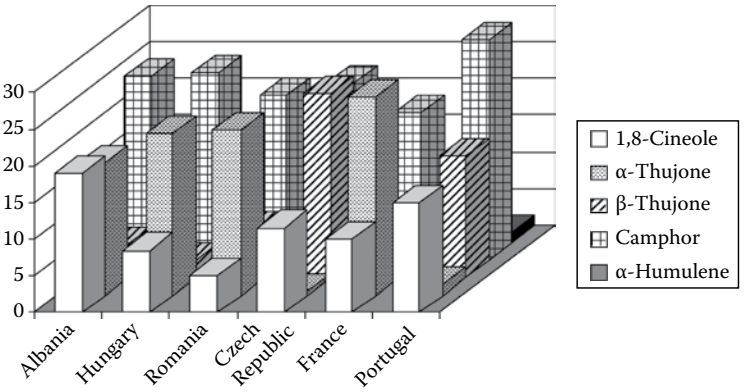

FIGURE 3.2 Composition of the essential oil of six *Salvia officinalis* origins.

of different publications, the picture is, however, not as clear as demonstrated by six *S. officinalis* origins in Figure 3.2 (Chalchat et al., 1998; Asllani, 2000). This might be due to the prevailing chemotype in a population, the variation between single plants, the time of sample collection, and the sample size. This is exemplarily shown by one *S. officinalis* population where the individuals varied in α-thujone, from 9% to 72%; β-thujone, from 2% to 24%; 1,8-cineole, from 4% to 18%; and camphor from 1% to 25%. The variation over 3 years and five harvests of one clone only ranged as follows: α-thujone 35%–72%, β-thujone 1%–7%, 1,8-cineole 8%–15%, and camphor 1%–18% (Bezzi, 1994; Bazina et al., 2002). But also all other (minor) compounds of the essential oil showed respective intraspecific variability (see, e.g., Giannouli and Kintzios, 2000).

S. fruticosa was principally understood to contain 1,8-cineole as main compound but at best traces of thujones, as confirmed by Putievsky et al. (1986) and Kanias et al. (1998). In a comparative study of several origins, Máthé et al. (1996) identified, however, a population with atypically high β-thujone similar to *S. officinalis*. Doubts on if this origin could be true *S. fruticosa* or a spontaneous hybrid of both species were resolved by extensive investigations on the phytochemical and genetic diversities of *S. fruticosa* in Crete (Karousou et al., 1998; Skoula et al., 1999). There, it was shown that all wild populations in western Crete consist of 1,8-cineole chemotypes only, whereas in the eastern part of the island, essential oils with up to 30% thujones, mainly β-thujone, could be observed. In central Crete, finally, mixed populations were found. A cluster analysis based on random amplification of polymorphic DNA (RAPD) patterns confirmed the genetic differences between the west and east Crete populations of *S. fruticosa* (Skoula et al., 1999).

A rather interesting example of diversity is *oregano*, which counts to the commercially most valued spices worldwide. More than 60 plant species are used under this common name showing similar flavor profiles characterized mainly by cymyl compounds, for example, carvacrol and thymol. With few exemptions, the majority of oregano species belong to the Lamiaceae and Verbenaceae families with the main genera *Origanum* and *Lippia* (Table 3.1). In 1989, almost all of the estimated 15,000 ton/year dried oregano originated from wild collection; today, some 7000 ha of *Origanum onites* are cultivated in Turkey alone (Baser, 2002); *O. onites* and other *Origanum* species are cultivated in Greece, Israel, Italy, Morocco, and other countries.

In comparison with sage, the genus *Origanum* is much smaller and consists of 43 species and 18 hybrids according to the actual classification (Skoula and Harborne, 2002) with main distribution areas around the Mediterranean. Some subspecies of *O. vulgare* only are also found in the temperate and arid zones of Eurasia up to China. Nevertheless, the genus is characterized by large morphological and phytochemical diversities (Kokkini et al., 1996; Baser, 2002; Skoula and Harborne, 2002).

The occurrence of several chemotypes is reported, for example, for commercially used *Origanum* species, from Turkey (Baser, 2002). In *O. onites*, two chemotypes are described, a carvacrol type and a linalool type. Additionally, a *mixed type* with both basic types mixed may occur. In

TABLE 3.1
Species Used Commercially in the World as Oregano

Family/Species	Commercial Name(s) Found in Literature
Labiatae	
Calamintha potosina Schaf.	Oregano de la sierra, oregano, origanum
Coleus amboinicus Lour. (syn. *C. aromaticus* Benth)	Oregano, oregano brujo, oregano de Cartagena, oregano de Espana, oregano Frances
Coleus aromaticus Benth.	Oregano de Espana, oregano, *Origanum*
Hedeoma floribunda Standl.	Oregano, *Origanum*
Hedeoma incona Torr.	Oregano
Hedeoma patens Jones	Oregano, *Origanum*
Hyptis albida HBK.	Oregano, *Origanum*
Hyptis americana (Aubl.) Urb. (*H. gonocephala* Gris.)	Oregano
Hyptis capitata Jacq.	Oregano, *Origanum*
Hyptis pectinata Poit.	Oregano, *Origanum*
Hyptis suaveolens (L.) Poit.	Oregano, oregano cimarron, *Origanum*
Monarda austromontana Epling	Oregano, *Origanum*
Ocimum basilicum L.	Oregano, *Origanum*
Origanum compactum Benth. (syn. *O. glandulosum* Salzm, ex Benth.)	Oregano, *Origanum*
Origanum dictamnus L. (*Majorana dictamnus* L.)	Oregano, *Origanum*
Origanum elongatum (Bonent) Emberger et Maire	Oregano, *Origanum*
Origanum floribundum Munby (*O. cinereum* Noe)	Oregano, *Origanum*
Origanum grosii Pau et Font Quer ex letswaart	Oregano, *Origanum*
Origanum majorana L.	Oregano
Origanum microphyllum (Benth) Vogel	Oregano, *Origanum*
Origanum onites L. (syn. *O. smyrneum* L.)	Turkish oregano, oregano, *Origanum*[a]
Origanum scabrum Boiss et Heldr. (syn. *O. pulchrum* Boiss et Heldr.)	Oregano, *Origanum*
Origanum syriacum L. var. *syriacum* (syn. *O. maru* L.)	Oregano, *Origanum*
Origanum vulgare L. ssp. *gracile* (Koch) letswaart (syn. *O. gracile* Koch, *O. tyttanthum* Gontscharov)	Oregano, *Origanum*
Origanum vulgare ssp. *hirtum* (Link) letswaart (syn *O. hirtum* Link)	Oregano, *Origanum*
Origanum vulgare ssp. *virens* (Hoffmanns et Link) letswaart (syn. *O. virens* Hoffmanns et Link)	Oregano, *Origanum*, oregano verde
Origanum vulgare ssp. *viride* (Boiss.) Hayek (syn. *O. viride*) Halacsy (syn. *O. heracleoticum* L.)	Greek oregano, oregano, *Origanum*[a]
Origanum vulgare L. ssp. *vulgare* (syn. *Thymus origanum* (L.) Kuntze)	Oregano, *Origanum*
Origanum vulgare L.	Oregano, orenga, Oregano de Espana
Poliomintha longiflora Gray	Oregano
Salvia sp.	Oregano
Satureja thymbra L.	Oregano cabruno, oregano, *Origanum*
Thymus capitatus (L.) Hoffmanns et Link (syn. *Coridothymus capitatus* (L.) Rchb.f.)	Spanish oregano, oregano, *Origanum*[a]
Verbenaceae	
Lantana citrosa (Small) Modenke	Oregano xiu, oregano, *Origanum*
Lantana glandulosissima Hayek	Oregano xiu, oregano silvestre, oregano, *Origanum*
Lantana hirsuta Mart et Gall.	Oreganillo del monte, oregano, *Origanum*
Lantana involucrata L.	Oregano, *Origanum*

(*Continued*)

TABLE 3.1 (*Continued*)
Species Used Commercially in the World as Oregano

Family/Species	Commercial Name(s) Found in Literature
Lantana purpurea (Jacq.) Benth. & Hook. (syn. *Lippia purpurea* Jacq.)	Oregano, *Origanum*
Lantana trifolia L.	Oregano, *Origanum*
Lantana velutina Mart. & Gal.	Oregano xiu, oregano, *Origanum*
Lippia myriocephala Schlecht. & Cham.	Oreganillo
Lippia affinis Schau.	Oregano
Lippia alba (Mill) N.E. Br. (syn. *L. involucrata* L.)	Oregano, *Origanum*
Lippia berlandieri Schau.	Oregano
Lippia cordiostegia Benth.	Oreganillo, oregano montes, oregano, *Origanum*
Lippia formosa T.S. Brandeg.	Oregano, *Origanum*
Lippia geisseana (R.A.Phil.) Soler.	Oregano, *Origanum*
Lippia graveolens HBK	Mexican oregano, oregano cimarron, oregano[a]
Lippia helleri Britton	Oregano del pais, oregano, *Origanum*
Lippia micromera Schau.	Oregano del pais, oregano, *Origanum*
Lippia micromera var. *helleri* (Britton) Moldenke	Oregano
Lippia origanoides HBK	Oregano, origano del pais
Lippia palmeri var. *spicata* Rose	Oregano
Lippia palmeri Wats.	Oregano, *Origanum*
Lippia umbellata Cav.	Oreganillo, oregano montes, oregano, *Origanum*
Lippia velutina Mart. et Galeotti	Oregano, *Origanum*
Rubiaceae	
Borreria sp.	Oreganos, oregano, *Origanum*
Scrophulariaceae	
Limnophila stolonifera (Blanco) Merr.	Oregano, *Origanum*
Apiaceae	
Eryngium foetidum L.	Oregano de Cartagena, oregano, *Origanum*
Asteraceae	
Coleosanthus veronicaefolius HBK	Oregano del cerro, oregano del monte, oregano del campo
Eupatorium macrophyllum L. (syn. *Hebeclinium macrophyllum* DC.)	Oregano, *Origanum*

[a] Oregano species with economic importance according to Lawrence (1984).

Turkey, two chemotypes of *Origanum majorana* are known, one contains *cis*-sabinene hydrate as chemotypical lead compound and is used as marjoram in cooking (*marjoramy*), while the other one contains carvacrol in high amounts and is used to distil *oregano oil* in a commercial scale. Variability of chemotypes continues also within the *marjoramy O. majorana*. Novak et al. (2002) detected in cultivated marjoram accessions additionally to *cis*-sabinene hydrate the occurrence of polymorphism of *cis*-sabinene hydrate acetate. Since this chemotype did not influence the sensorial impression much, this chemotype was not eliminated in breeding, while an *off-flavor* chemotype would have been certainly eliminated in its cultivation history. In natural populations of *O. majorana* from Cyprus besides the *classical cis*-sabinene hydrate type, a chemotype with α-terpineol as main compound was also detected (Novak et al., 2008). The two extreme *off-flavor* chemotypes in *O. majorana*, carvacrol and α-terpineol chemotypes, are not to be found anywhere in cultivated marjoram, demonstrating one of the advantages of cultivation in delivering homogeneous qualities.

The second *oregano* of commercial value—mainly used in the Americas—is *Mexican oregano* (*Lippia graveolens* HBK., Verbenaceae) endemic to California, Mexico, and throughout Central America (Fischer, 1998). Due to wild harvesting, only a few published data show essential oil contents largely ranging from 0.3% to 3.6%. The total number of up-to-now-identified essential oil compounds comprises almost 70 with the main constituents thymol (3.1%–80.6%), carvacrol (0.5%–71.2%), 1,8-cineole (0.1%–14%), and *p*-cymene (2.7%–28.0%), followed by, for example, myrcene, γ-terpinene, and the sesquiterpene caryophyllene (Lawrence, 1984; Dominguez et al., 1989; Uribe-Hernández et al., 1992; Fischer et al., 1996; Vernin et al., 2001).

In a comprehensive investigation of wild populations of *L. graveolens* collected from the hilly regions of Guatemala, three different essential oil chemotypes could be identified, a thymol, a carvacrol, and an absolutely irregular type (Fischer et al., 1996). Within the thymol type, contents of up to 85% thymol in the essential oil could be obtained and only traces of carvacrol. The irregular type has shown a very uncommon composition where no compound exceeds 10% of the oil, and also phenylpropenes, for example, eugenol and methyl eugenol, were present (Fischer et al., 1996; Fischer, 1998). In Table 3.2, a comparison of recent data is given including *Lippia alba*, commonly called *oregano* or *oregano del monte*, although carvacrol and thymol are absent from the essential oil of this species. In Guatemala, two different chemotypes were found within *L. alba*: a myrcenone and a citral type (Fischer et al., 2004). Besides it, a linalool, a carvone, a camphor (1,8-cineole), and a limonene–piperitone chemotype have been described (Dellacassa et al., 1990; Pino et al., 1997; Frighetto et al., 1998; Senatore and Rigano, 2001).

Chemical diversity is of special interest if on genus or species level both terpenes and phenylpropenes can be found in the essential oil. Most Lamiaceae preferentially accumulate mono- and sesquiterpenes in their volatile oils, but some genera produce oils also rich in phenylpropenes, among these *Ocimum* sp. and *Perilla* sp.

The genus *Ocimum* comprises over 60 species, of which *Ocimum gratissimum* and *O. basilicum* are of high economic value. Biogenetic studies on the inheritance of *Ocimum* oil constituents were reported by Khosla et al. (1989) and an *O. gratissimum* strain named *clocimum* containing 65% of eugenol in its oil was described by Bradu et al. (1989). A number of different chemotypes of basil (*O. basilicum)* have been identified and classified (Vernin et al., 1984; Marotti et al., 1996) containing up to 80% linalool, up to 21.5% 1,8-cineole, 0.3%–33.0% eugenol, and also the presumably toxic compounds methyl chavicol (estragole) and methyl eugenol in concentrations close to 50% (Elementi et al., 2006; Macchia et al., 2006).

Perilla frutescens can be classified in several chemotypes as well according to the main monoterpene components perillaldehyde, elsholtzia ketone, or perilla ketones and on the other side phenylpropanoid types containing myristicin, dillapiole, or elemicin (Koezuka et al., 1986). A comprehensive presentation on the chemotypes and the inheritance of the mentioned compounds was given by this author in Hay and Waterman (1993). In the referred last two examples, not only the sensorial but also the toxicological properties of the essential oil compounds are decisive for the (further) commercial use of the respective species" biodiversity.

Although the Labiatae family plays an outstanding role as regards the chemical polymorphism of essential oils, also in other essential oils containing plant families and genera, a comparable phytochemical diversity can be observed.

3.2.2.2 Asteraceae (Compositae)

Only a limited number of genera of the Asteraceae are known as essential oil plants, among them *Tagetes*, *Achillea*, and *Matricaria*. The genus *Tagetes* comprises actually 55 species, all of them endemic to the American continents with the center of biodiversity between 30° northern and 30° southern latitude. One of the species largely used by the indigenous population is *pericon* (*T. lucida* Cav.), widely distributed over the highlands of Mexico and Central America (Stanley and Steyermark, 1976). In contrast to almost all other *Tagetes* species characterized by the content of tagetones, this species contains phenylpropenes and terpenes. A detailed study on its diversity

TABLE 3.2
Main Essential Oil Compounds of *Lippia graveolens* and *L. alba* According to Recent Data

	L. graveolens					*L. alba*			
	Fischer et al. (1996)			Senatore and Rigano (2001)	Vernin et al. (2001)	Fischer (1998)		Senatore and Rigano (2001)	Lorenzo et al. (2001)
	Guatemala					Guatemala			
Compound	**Thymol-Type**	**Carvacrol-Type**	**Irregular Type**	**Guatemala**	**El-Salvador**	**Myrcenone-Type**	**Cineole-Type**	**Guatemala**	**Uruguay**
Myrcene	1.3	1.9	2.7	1.1	t	6.5	1.7	0.2	0.8
p-Cymene	2.7	6.9	2.8	5.5	2.1	t	t	0.7	n.d.
1,8-Cineole	0.1	0.6	5.0	2.1	t	t	**22.8**	**14.2**	1.3
Limonene	0.2	0.3	1.5	0.8	t	1.0	3.2	**43.6**	2.9
Linalool	0.7	1.4	3.8	0.3	t	4.0	2.4	1.2	**55.3**
Myrcenon	n.d.	n.d.	n.d.	n.d.	n.d.	**54.6**	3.2	n.d.	n.d.
Piperitone	n.d.	n.d.	n.d.	n.d.	n.d.	t	t	**30.6**	n.d.
Thymol	**80.6**	**19.9**	6.8	**31.6**	7.3	n.d.	n.d.	n.d.	n.d.
Carvacrol	1.3	**45.2**	1.1	0.8	**71.2**	n.d.	n.d.	n.d.	n.d.
β-Caryophyllene	2.8	3.5	8.7	4.6	9.2	2.6	1.2	1.0	9.0
α-Humulene	1.9	2.3	5.7	3.0	5.0	0.7	t	0.6	0.9
Caryophyll.-ox.	0.3	0.8	3.3	4.8	t	1.8	3.0	1.1	0.6
Z-Dihydrocarvon/*Z*-Ocimenone	n.d.	n.d.	n.d.	n.d.	n.d.	13.1	0.6	0.1	0.8
E-Dihydrocarvon	n.d.	n.d.	n.d.	n.d.	n.d.	4.9	n.d.	t	1.2

Note: n.d., Not detectable; t, traces. Main compounds in bold.

TABLE 3.3
Main Compounds of the Essential Oil of Selected *Tagetes lucida* Types (in% of dm)

Substance	Anethole Type (2)	Estragole Type (8)	Methyleugenol Type (7)	Nerolidol Type (5)	Mixed Type
Linalool	0.26	0.69	1.01	Tr.	3.68
Estragole	11.57	**78.02**	8.68	3.23	**24.28**
Anethole	**73.56**	0.75	0.52	Tr.	**30.17**
Methyleugenol	1.75	5.50	**79.80**	17.76	**17.09**
β-Caryophyllene	0.45	1.66	0.45	2.39	0.88
Germacrene D	2.43	2.89	1.90	Tr.	5.41
Methylisoeugenol	1.42	2.78	2.00	Tr.	3.88
Nerolidol	0.35	0.32	0.31	**40.52**	1.24
Spathulenol	0.10	0.16	0.12	Tr.	0.23
Carophyllene oxide	0.05	0.27	0.45	10.34	0.53

Note: Location of origin in Guatemala: (2) Cabrican/Quetzaltenango, (5) La Fuente/Jalapa, (7) Joyabaj/El Quiche, (8) Sipacapa/S. Marcos, Mixed Type: Taltimiche/San Marcos. Main compounds in bold.

in Guatemala resulted in the identification of several eco- and chemotypes (Table 3.3): anethole, methyl chavicol (estragole), methyl eugenol, and one sesquiterpene type producing higher amounts of nerolidol (Bicchi et al., 1997; Goehler, 2006). The distribution of the three main phenylpropenes in six populations is illustrated in Figure 3.3. In comparison with the plant materials investigated by Ciccio (2004) and Marotti et al. (2004) containing oils with 90%–95% estragole, only the germplasm collection of Guatemaltecan provenances (Goehler, 2006) allows to select individuals with high anethole but low to very low estragole or methyleugenol content—or with interestingly high nerolidol content, as mentioned earlier.

The genus *Achillea* is widely distributed over the northern hemisphere and consists of approximately 120 species, of which the *Achillea millefolium* aggregate (yarrow) represents a polyploid complex of allogamous perennials (Saukel and Länger, 1992; Vetter and Franz, 1996). The different taxa of the recent classification (*minor species* and *subspecies*) are morphologically and chemically to a certain extent distinct and only the diploid taxa *Achillea asplenifolia* and *A. roseoalba* as well as the tetraploids *A. collina* and *Achillea ceretanica* are characterized by proazulens, for example, achillicin, whereas the other taxa, especially 6× and 8×, contain eudesmanolides, longipinenes, germacranolides, and/or guajanolid peroxides (Table 3.4). The intraspecific variation in the proazulene content ranged from traces up to 80%; other essential oil components of the azulenogenic species are, for example,

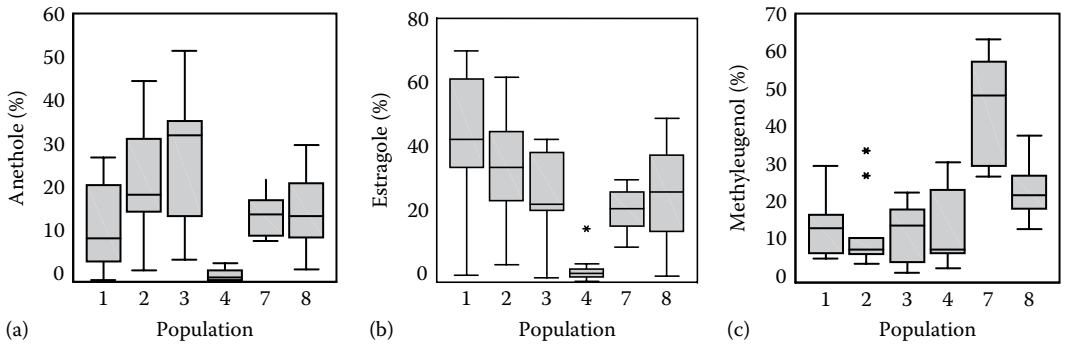

FIGURE 3.3 Variability of (a) anethole, (b) methyl chavicol (estragole), and (c) methyl eugenol in the essential oil of six *Tagetes lucida*—populations from Guatemala. * indicates erratic individuals.

TABLE 3.4
Taxa within the *Achillea-Millefolium*-Group (Yarrow)

Taxon	Ploidy Level	Main Compounds
A. setacea W. et K.	2×	Rupicoline
A. aspleniifolia Vent.	2× (4×)	**7,8-Guajanolide**
		Artabsin-derivatives
		3-Oxa-Guajanolide
A. roseo-alba Ehrend.	2×	Artabsin-derivatives
		3-Oxaguajanolide
		Matricinderivatives
A. collina Becker	4×	Artabsin-derivatives
		3-Oxaguajanolide
		Matricinderivatives
		Matricarinderivatives
A. pratensis Saukel u. Länger	4×	Eudesmanolides
A. distans ssp. Distans W. et K.	6×	Longipinenones
A. distans ssp. *styriaca*	4×	
A. tanacetifolia (stricta) W. et K.	6×	
A. mill. ssp. *sudetica*	6×	Guajanolidperoxide
A. mill. ssp. Mill. L.	6×	
A. pannonica Scheele	8× (6×)	Germacrene
		Guajanolidperoxide

Source: Franz, Ch., 2013. In *Handbuch des Arznei- u. Gewuerzpflanzenbaus*, Vol. 5, pp. 453–463. Saluplanta, Bernburg.
Note: Substances in bold are proazulenes.

α- and β-pinene, borneol, camphor, sabinene, or caryophyllene (Kastner et al., 1992). The frequency distribution of proazulene individuals among two populations is shown in Figure 3.4.

Crossing experiments resulted in proazulene being a recessive character of di- and tetraploid *Achillea* spp. (Vetter et al., 1997) similar to chamomile (Franz, 1993a,b). Finally, according to Steinlesberger (2002) also a plant-to-plant variation in the enantiomers of, for example, α- and β-pinene as well as sabinene exists in yarrow oils, which makes it even more complicated to use phytochemical characters for taxonomical purposes.

Differences in the essential oil content and composition of chamomile flowers (*Matricaria recutita*) have long been recognized due to the fact that the distilled oil is either dark blue, green, or yellow,

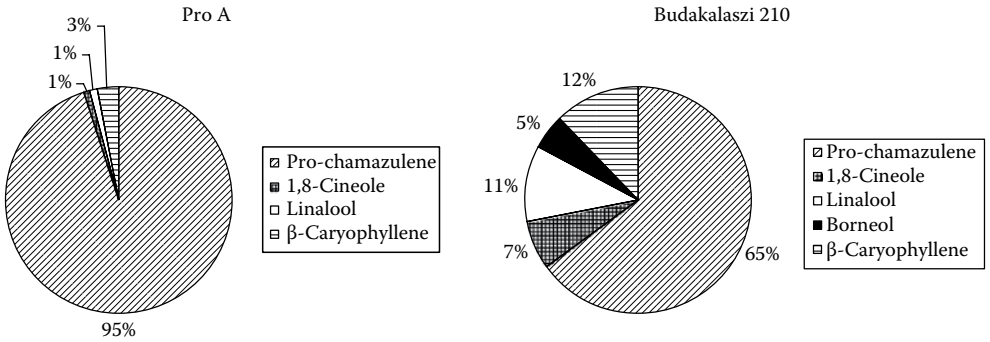

FIGURE 3.4 Frequency distribution of proazulene individuals among two *Achillea* sp. populations.

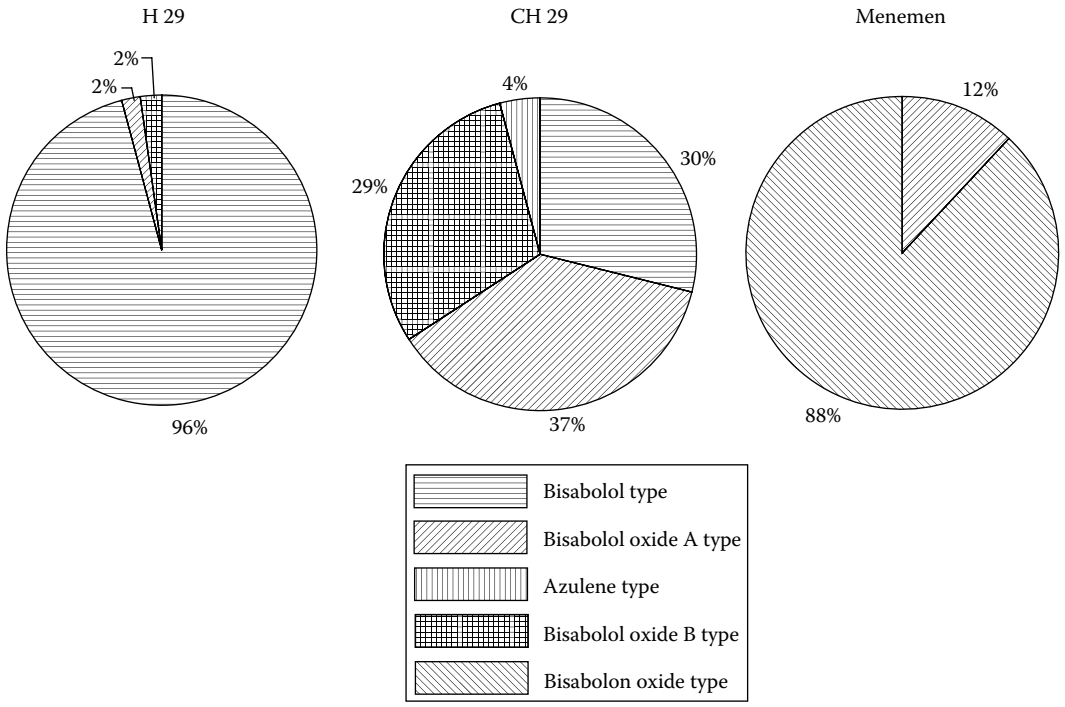

FIGURE 3.5 Frequency distribution of chemotypes in three varieties/populatios of chamomile (*Matricaria recutita* (L.) Rauschert).

depending on the prochamazulene content (matricin as prochamazulene in chamomile is transformed to the blue-colored artifact chamazulene during the distillation process). Recognizing also the great pharmacological potential of the bisabolols, a classification into the chemotypes (−)-α-bisabolol, (−)-α-bisabololoxide A, (−)-α-bisabololoxide B, (−)-α-bisabolonoxide (A), and (pro)chamazulene was made by Franz (1982, 1989a). Examining the geographical distribution revealed a regional differentiation, where an α-bisabolol—(pro) chamazulene population was identified on the Iberian peninsula; mixed populations containing chamazulene, bisabolol, and bisabololoxides A/B are most frequent in Central Europe, and prochamazulene—free bisabolonoxide populations are indigenous to southeast Europe and minor Asia. In the meantime, Wogiatzi et al. (1999) have shown for Greece and Taviani et al. (2002) for Italy a higher diversity of chamomile including α-bisabolol types. This classification of populations and chemotypes was extended by analyzing populations at the level of individual plants (Schröder, 1990) resulting in the respective frequency distributions (Figure 3.5).

In addition, the range of essential oil components in the chemotypes of one Central European population is shown in Table 3.5 (Franz, 2000).

Data on inter- and intraspecific variation of essential oils are countless, and recent reviews are known for a number of genera published, for example, in the series "Medicinal and Aromatic Plants—Industrial Profiles" (Harwood Publications, Taylor & Francis, CRC Press, respectively).

The generally observed quantitative and qualitative variations in essential oils draw the attention *i.a.* to appropriate random sampling for getting valid information on the chemical profile of a species or population. As concerns quantitative variations of a certain pattern or substance, Figure 3.6 shows exemplarily the bisabolol content of two chamomile populations depending on the number of individual plants used for sampling. At small numbers, the mean value oscillates strongly, and only after at least 15–20 individuals the range of variation becomes acceptable. Quite different appears the situation at qualitative differences, that is, *either–or variations* within populations or taxa, for example, carvacrol/thymol, α-/β-thujone/1,8-cineole/camphor, or monoterpenes/phenylpropenes.

TABLE 3.5
Grouping within a European Spontaneous Chamomile, Figures in % of Terpenoids in the Essential Oil of the Flower Heads

	Chamazulen	α-Bisabolol	α-B.-Oxide A	α-B.-Oxide B
α-Bisabolol-type				
Range	2.5–35.2	58.8–92.1	n.d.–1.0	n.d.–3.2
Mean	23.2	**68.8**	n.d.	n.d.
α-Bisabololoxide A-type				
Range	6.6–31.2	0.5–12.3	31.7–66.7	1.9–22.4
Mean	21.3	2.1	**53.9**	11.8
α-Bisabololoxide B-type				
Range	7.6–24.2	0.8–6.5	1.6–4.8	61.6–80.5
Mean	16.8	2.0	2.6	**72.2**
Chamazulene-type				
Range	76.3–79.2	5.8–8.3	n.d.–0.8	n.d.–2.6
Mean	**77.8**	7.1	n.d.	n.d.

Source: Franz, Ch., 2000. *Biodiversity and Random Sampling in Essential Oil Plants*. Lecture 31st ISEO, Hamburg, Germany.
Note: Main compounds in bold. n.d., not detected (determined).

Any random sample may give nonspecific information only on the principal chemical profile of the respective population provided that the sample is representative. This depends on the number of chemotypes, their inheritance, and frequency distribution within the population, and generally speaking, no less than 50 individuals are needed for that purpose, as it can be derived from the comparison of chemotypes in a *Thymus vulgaris* population (Figure 3.7).

The overall high variation in essential oil compositions can be explained by the fact that quite different products might be generated by small changes in the synthase sequences only. On the other hand, different synthases may be able to produce the same substance in systematically distant taxa. The different origin of such substances can be identified by, for example, the $^{12}C/^{13}C$ ratio (Mosandl, 1993). Bazina et al. (2002) stated, "Hence, a simple quantitative analysis of the essential oil composition is not necessarily appropriate for estimating genetic proximity even in closely related taxa."

3.3 IDENTIFICATION OF SOURCE MATERIALS

As illustrated by the previous paragraph, one of the crucial points of using plants as sources for essential oils is their heterogeneity. A first prerequisite for reproducible compositions is therefore an unambiguous botanical identification and characterization of the starting material. The first approach is the classical taxonomical identification of plant materials based on macro- and micromorphological features of the plant. The identification is followed by phytochemical analysis that may contribute to species identification as well as to the determination of the quality of the essential oil. This approach is now complemented by DNA-based identification.

DNA is a long polymer of nucleotides, the building units. One of four possible nitrogenous bases is part of each nucleotide, and the sequence of the bases on the polymer strand is characteristic for each living individual. Some regions of the DNA, however, are conserved on the species or family level and can be used to study the relationship of taxa (Taberlet et al., 1991; Wolfe and Liston, 1998). DNA sequences conserved within a taxon but different between taxa can therefore be used to identify a taxon (*DNA barcoding*) (Hebert et al., 2003; Kress et al., 2005). A DNA-barcoding consortium was founded in 2004 with the ambitious goal to build a barcode library for all eukaryotic life in the next 20 years (Ratnasingham and Hebert, 2007). New sequencing technologies (454, Solexa, SOLiD) enable a fast and

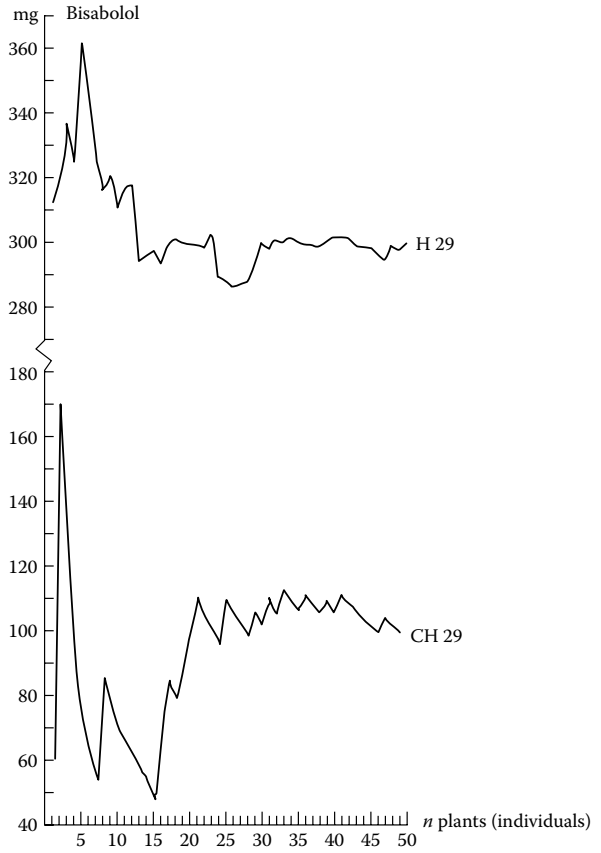

FIGURE 3.6 (−)-α-Bisabolol-content (mg/100 g crude drug) in two chamomile (*Matricaria recutita*) populations: mean value in dependence of the number of individuals used for sampling.

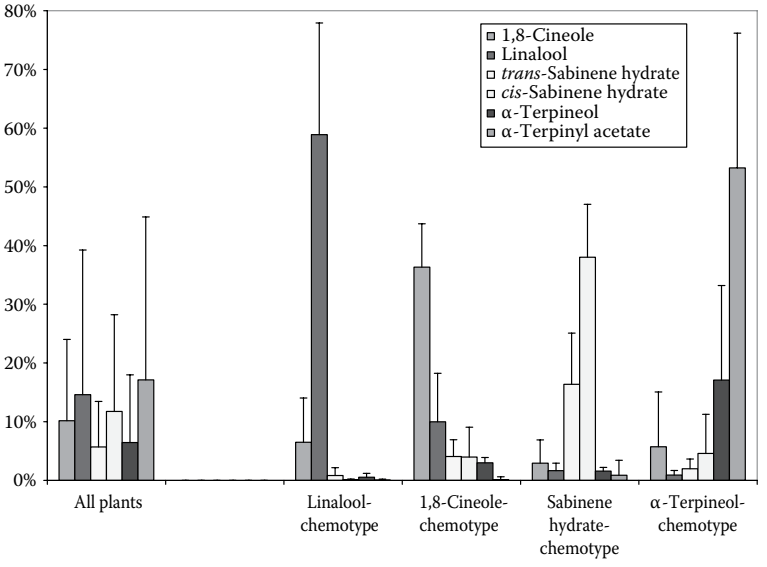

FIGURE 3.7 Mean values of the principal essential compounds of a *Thymus vulgaris* population (left) in comparison to the mean values of the chemotypes within the same population.

representative analysis, but will be applied due to their high costs in the moment only in the next phase of DNA barcoding (Frezal and Leblois, 2008). DNA barcoding of animals has already become a routine task. DNA barcoding of plants, however, is still not trivial and a scientific challenge (Pennisi, 2007).

Besides sequence information–based approaches, multilocus DNA methods (RAPD, amplified fragment length polymorphism, etc.) are complementing in resolving complicated taxa and can become a barcode for the identification of populations and cultivars (Weising et al., 2005). With multilocus DNA methods, it is furthermore possible to tag a specific feature of a plant of which the genetic basis is still unknown. This approach is called molecular markers (in *sensu strictu*) because they mark the occurrence of a specific trait like a chemotype or flower color. The gene regions visualized, for example, on an agarose gel are not the specific gene responsible for a trait but are located on the genome in the vicinity of this gene and therefore co-occur with the trait and are absent when the trait is absent. An example for such an inexpensive and fast polymerase chain reaction system was developed by Bradbury et al. (2005) to distinguish fragrant from nonfragrant rice cultivars. If markers would be developed for chemotypes in essential oil plants, species identification by DNA and the determination of a chemotype could be performed in one step.

Molecular biological methods to identify species are nowadays routinely used in feed- and foodstuffs to identify microbes, animals, and plants. Especially the discussion about traceability of genetically modified organisms (GMOs) throughout the complete chain ("from the living organism to the supermarket") has sped up research in this area (Auer, 2003; Miraglia et al., 2004). One advantage of molecular biological methods is the possibility to be used in a number of processed materials like fatty oil (Pafundo et al., 2005) or even solvent extracts (Novak et al., 2007). The presence of minor amounts of DNA in an essential oil cannot be excluded *a priori*, although distillation as separation technique would suggest the absence of DNA. However, small plant or DNA fragments could distill over or the essential oil could come in contact with plant material after distillation.

3.4 GENETIC AND PROTEIN ENGINEERING

Genetic engineering is defined as the direct manipulation of the genes of organisms by laboratory techniques, not to be confused with the indirect manipulation of genes in traditional (plant) breeding. *Transgenic or GMOs* are organisms (bacteria, plants, etc.) that have been engineered with single or multiple genes (either from the same species or from a different species), using contemporary molecular biology techniques. These are organisms with improved characteristics, in plants, for example, with resistance or tolerance to biotic or abiotic stresses such as insects, disease, drought, salinity, and temperature. Another important goal in improving agricultural production conditions is to facilitate weed control by transformed plant resistant to broadband herbicides like glufosinate. Peppermint has been successfully transformed with the introduction of the bar gene, which encodes phosphinothricin acetyltransferase, an enzyme inactivating glufosinate ammonium or the ammonium salt of glufosinate, phosphinothricin, making the plant insensitive to the systemic, broadspectrum herbicide Roundup (*Roundup Ready mint*) (Li et al., 2001).

A first step in genetic engineering is the development and optimization of transformation (gene transfer) protocols for the target species. Such optimized protocols exist for essential oil plants such as lavandin (*Lavandula* × *intermedia*; Dronne et al., 1999), spike lavender (*Lavandula latifolia*; Nebauer et al., 2000), and peppermint (*M. piperita*; Diemer et al., 1998; Niu et al., 2000).

In spike lavender, an additional copy of the 1-deoxy-D-xylulose-5-phosphate synthase gene, the first enzymatic step in the methylerythritol phosphate (MEP) pathway leading to the precursors of monoterpenes, from *Arabidopsis thaliana*, was introduced and led to an increase of the essential oil of the leaves of up to 360% and of the essential oil of flowers of up to 74% (Munoz-Bertomeu et al., 2006).

In peppermint, many different steps to alter essential oil yield and composition were already targeted (reviewed by Wildung and Croteau, 2005; Table 3.6). The overexpression of deoxyxylulose phosphate reductoisomerase (DXR), the second step in the MEP pathway, increased the essential oil yield by approximately 40% tested under field conditions (Mahmoud and Croteau, 2001). The overexpression of

TABLE 3.6
Essential Oil Composition and Yield of Transgenic Peppermint Transformed with Genes Involved in Monoterpene Biosynthesis

Gene	Method	Limonene	Mentho-Furan	Pulegone	Menthone	Menthol	Oil Yield (lb/acre)
WT	—	1.7	4.3	2.1	20.5	44.5	97.8
DXR	Overexpress	1.6	3.6	1.8	19.6	45.6	137.9
MFS	Antisense	1.7	1.2	0.4	22.7	45.2	109.7
l-3-h	Cosuppress	74.7	0.4	0.1	4.1	3.0	99.6

Source: Wildung, M.R. and R.B. Croteau, 2005. *Transgenic Res.*, 14: 365.

Note: DXR, Deoxyxylulose phosphate reductoisomerase; l-3-h, limonene-3-hydroxylase; MFS, menthofuran synthase; WT, wild type.

geranyl diphosphate synthase leads to a similar increase of the essential oil. Menthofuran, an undesired compound, was downregulated by an antisense method (a method to influence or block the activity of a specific gene). Overexpression of the menthofuran antisense RNA was responsible for an improved oil quality by reducing both menthofuran and pulegone in one transformation step (Mahmoud and Croteau, 2003). The ability to produce a peppermint oil with a new composition was demonstrated by Mahmoud et al. (2004) by upregulating limonene by cosuppression of limonene-3-hydroxylase, the enzyme responsible for the transformation of (−)-limonene to (−)-*trans*-isopiperitenol *en route* to menthol.

Protein engineering is the application of scientific methods (mathematical and laboratory methods) to develop useful or valuable proteins. There are two general strategies for protein engineering, random mutagenesis and rational design. In rational design, detailed knowledge of the structure and function of the protein is necessary to make desired changes by site-directed mutagenesis, a technique already well developed. An impressive example of the rational design of monoterpene synthases was given by Kampranis et al. (2007) who converted a 1,8-cineole synthase from *S. fruticosa* into a synthase producing sabinene, the precursor of α- and β-thujones with a minimum number of substitutions. They went also a step further and converted this monoterpene synthase into a sesquiterpene synthase by substituting a single amino acid that enlarged the cavity of the active site enough to accommodate the larger precursor of the sesquiterpenes, farnesyl pyrophosphate.

3.5 RESOURCES OF ESSENTIAL OILS: WILD COLLECTION OR CULTIVATION OF PLANTS

The raw materials for producing essential oil are resourced either from collecting them in nature (*wild collection*) or from cultivating the plants (Table 3.7).

3.5.1 Wild Collection and Sustainability

Since prehistoric times, humans have gathered wild plants for different purposes; among them are aromatic, essential oil–bearing species used as culinary herbs, spices, flavoring agents, and fragrances. With increasing demand of standardized, homogeneous raw material in the industrial societies, more and more wild species have been domesticated and systematically cultivated. Nevertheless, a high number of species are still collected from the wild due to the fact that

- Many plants and plant products are used for the subsistence of the rural population.
- Small quantities of the respective species are requested at the market only which make a systematic cultivation not profitable.
- Some species are difficult to cultivate (slow growth rate and requirement of a special microclimate).

TABLE 3.7
Important Essential Oil-Bearing Plants—Common and Botanical Names Including Family, Plant Parts Used, Raw Material Origin, and Trade Quantities of the Essential Oil

Trade Name	Species	Plant Family	Used Plant Part(s)	Wild Collection/ Cultivation	Trade Quantities[a]
Ambrette seed	*Hibiscus abelmoschus* L.	Malvaceae	Seed	Cult	LQ
Amyris	*Amyris balsamifera* L.	Rutaceae	Wood	Wild	LQ
Angelica root	*Angelica archangelica* L.	Apiaceae	Root	Cult	LQ
Anise seed	*Pimpinella anisum* L.	Apiaceae	Fruit	Cult	LQ
Armoise	*Artemisia herba-alba* Asso.	Asteraceae	Herb	Cult/wild	LQ
Asafoetida	*Ferula assa-foetida* L.	Apiaceae	Resin	Wild	LQ
Basil	*Ocimum basilicum* L.	Lamiaceae	Herb	Cult	LQ
Bay	*Pimenta racemosa* Moore	Myrtaceae	Leaf	Cult	LQ
Bergamot	*Citrus aurantium* L. ssp. *bergamia* (Risso et Poit.) Engl.	Rutaceae	Fruit peel	Cult	MQ
Birch tar	*Betula pendula* Roth. (syn. *Betula verrucosa* Erhart. *Betula alba* sensu H.J.Coste. non L.)	Betulaceae	Bark/ wood	Wild	LQ
Buchu leaf	*Agathosma betulina* (Bergius) Pillans. *A. crenulata* (L.) Pillans	Rutaceae	Leaf	Wild	LQ
Cade	*Juniperus oxycedrus* L.	Cupressaceae	Wood	Wild	LQ
Cajuput	*Melaleuca leucandendron* L.	Myrtaceae	Leaf	Wild	LQ
Calamus	*Acorus calamus* L.	Araceae	Rhizome	Cult/wild	LQ
Camphor	*Cinnamomum camphora* L. (Sieb.)	Lauraceae	Wood	Cult	LQ
Cananga	*Cananga odorata* Hook. f. et Thoms.	Annonaceae	Flower	Wild	LQ
Caraway	*Carum carvi* L.	Apiaceae	Seed	Cult	LQ
Cardamom	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	Seed	Cult	LQ
Carrot seed	*Daucus carota* L.	Apiaceae	Seed	Cult	LQ
Cascarilla	*Croton eluteria* (L.) W.Wright	Euphorbiaceae	Bark	Wild	LQ
Cedarwood, Chinese	*Cupressus funebris* Endl.	Cupressaceae	Wood	Wild	MQ
Cedarwood, Texas	*Juniperus mexicana* Schiede	Cupressaceae	Wood	Wild	MQ
Cedarwood, Virginia	*Juniperus virginiana* L.	Cupressaceae	Wood	Wild	MQ
Celery seed	*Apium graveolens* L.	Apiaceae	Seed	Cult	LQ
Chamomile	*Matricaria recutita* L.	Asteraceae	Flower	Cult	LQ
Chamomile, Roman	*Anthemis nobilis* L.	Asteraceae	Flower	Cult	LQ
Chenopodium	*Chenopodium ambrosioides* (L.) Gray	Chenopodiaceae	Seed	Cult	LQ
Cinnamon bark, Ceylon	*Cinnamomum zeylanicum* Nees	Lauraceae	Bark	Cult	LQ
Cinnamon bark, Chinese	*Cinnamomum cassia* Blume	Lauraceae	Bark	Cult	LQ

(Continued)

TABLE 3.7 (*Continued*)
Important Essential Oil-Bearing Plants—Common and Botanical Names Including Family, Plant Parts Used, Raw Material Origin, and Trade Quantities of the Essential Oil

Trade Name	Species	Plant Family	Used Plant Part(s)	Wild Collection/ Cultivation	Trade Quantities[a]
Cinnamon leaf	*Cinnamomum zeylanicum* Nees	Lauraceae	Leaf	Cult	LQ
Citronella, Ceylon	*Cymbopogon nardus* (L.) W. Wats.	Poaceae	Leaf	Cult	HQ
Citronella, Java	*Cymbopogon winterianus* Jowitt.	Poaceae	Leaf	Cult	HQ
Clary sage	*Salvia sclarea* L.	Lamiaceae	Flowering herb	Cult	MQ
Clove buds	*Syzygium aromaticum* (L.) Merill et L.M. Perry	Myrtaceae	Leaf/bud	Cult	LQ
Clove leaf	*Syzygium aromaticum* (L.) Merill et L.M. Perry	Myrtaceae	Leaf	Cult	HQ
Coriander	*Coriandrum sativum* L.	Apiaceae	Fruit	Cult	LQ
Cornmint	*Mentha canadensis* L. (syn. *M. arvensis* L. f. *piperascens* Malinv. ex Holmes; *M. arvensis* L. var. *glabrata. M. haplocalyx* Briq.; *M. sachalinensis* [Briq.] Kudo)	Lamiaceae	Leaf	Cult	HQ
Cumin	*Cuminum cyminum* L.	Apiaceae	Fruit	Cult	LQ
Cypress	*Cupressus sempervirens* L.	Cupressaeae	Leaf/twig	Wild	LQ
Davana	*Artemisia pallens* Wall.	Asteraceae	Flowering herb	Cult	LQ
Dill	*Anethum graveolens* L.	Apiaceae	Herb/fruit	Cult	LQ
Dill, India	*Anethum sowa* Roxb.	Apiaceae	Fruit	Cult	LQ
Elemi	*Canarium luzonicum* Miq.	Burseraceae	Resin	Wild	LQ
Eucalyptus	*Eucalyptus globulus* Labill.	Myrtaceae	Leaf	Cult/wild	HQ
Eucalyptus, lemon-scented	*Eucalyptus citriodora* Hook.	Myrtaceae	Leaf	Cult/wild	HQ
Fennel bitter	*Foeniculum vulgare* Mill. ssp. *vulgare* var. *vulgare*	Apiaceae	Fruit	Cult	LQ
Fennel sweet	*Foeniculum vulgare* Mill. ssp. *vulgare* var. *dulce*	Apiaceae	Fruit	Cult	LQ
Fir needle, Canadian	*Abies balsamea* Mill.	Pinaceae	Leaf/twig	Wild	LQ
Fir needle, Siberian	*Abies sibirica* Ledeb.	Pinaceae	Leaf/twig	Wild	LQ
Gaiac	*Guaiacum officinale* L.	Zygophyllaceae	Resin	Wild	LQ
Galbanum	*Ferula galbaniflua* Boiss. *F. rubricaulis* Boiss.	Apiaceae	Resin	Wild	LQ
Garlic	*Allium sativum* L.	Alliaceae	Bulb	Cult	LQ
Geranium	*Pelargonium* spp.	Geraniaceae	Leaf	Cult	MQ
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Rhizome	Cult	LQ

(Continued)

TABLE 3.7 (*Continued*)
Important Essential Oil-Bearing Plants—Common and Botanical Names Including Family, Plant Parts Used, Raw Material Origin, and Trade Quantities of the Essential Oil

Trade Name	Species	Plant Family	Used Plant Part(s)	Wild Collection/ Cultivation	Trade Quantities[a]
Gingergrass	*Cymbopogon martinii* (Roxb.) H. Wats var. *sofia* Burk	Poaceae	Leaf	Cult/wild	
Grapefruit	*Citrus* × *paradisi* Macfad.	Rutaceae	Fruit peel	Cult	LQ
Guaiacwood	*Bulnesia sarmienti* L.	Zygophyllaceae	Wood	Wild	MQ
Gurjum	*Dipterocarpus* spp.	Dipterocarpaceae	Resin	Wild	LQ
Hop	*Humulus lupulus* L.	Cannabaceae	Flower	Cult	LQ
Hyssop	*Hyssopus officinalis* L.	Lamiaceae	Leaf	Cult	LQ
Juniper berry	*Juniperus communis* L.	Cupressaceae	Fruit	Wild	LQ
Laurel leaf	*Laurus nobilis* L.	Lauraceae	Leaf	Cult/wild	LQ
Lavandin	*Lavandula angustifolia* Mill. × *L. latifolia* Medik.	Lamiaceae	Leaf	Cult	HQ
Lavender	*Lavandula angustifolia* Miller	Lamiaceae	Leaf	Cult	MQ
Lavender, Spike	*Lavandula latifolia* Medik.	Lamiaceae	Flower	Cult	LQ
Lemon	*Citrus limon* (L.) Burman fil.	Rutaceae	Fruit peel	Cult	HQ
Lemongrass, Indian	*Cymbopogon flexuosus* (Nees ex Steud.) H. Wats.	Poaceae	Leaf	Cult	LQ
Lemongrass, West Indian	*Cymbopogon citratus* (DC.) Stapf	Poaceae	Leaf	Cult	LQ
Lime distilled	*Citrus aurantiifolia* (Christm. et Panz.) Swingle	Rutaceae	Fruit	Cult	HQ
Litsea cubeba	*Litsea cubeba* C.H. Persoon	Lauraceae	Fruit/leaf	Cult	MQ
Lovage root	*Levisticum officinale* Koch	Apiaceae	Root	Cult	LQ
Mandarin	*Citrus reticulata* Blanco	Rutaceae	Fruit peel	Cult	MQ
Marjoram	*Origanum majorana* L.	Lamiaceae	Herb	Cult	LQ
Mugwort common	*Artemisia vulgaris* L.	Asteraceae	Herb	Cult/wild	LQ
Mugwort, Roman	*Artemisia pontica* L.	Asteraceae	Herb	Cult/wild	LQ
Myrtle	*Myrtus communis* L.	Myrtaceae	Leaf	Cult/wild	LQ
Neroli	*Citrus aurantium* L. ssp. *aurantium*	Rutaceae	Flower	Cult	LQ
Niaouli	*Melaleuca viridiflora*	Myrtaceae	Leaf	Cult/wild	LQ
Nutmeg	*Myristica fragrans* Houtt.	Myristicaceae	Seed	Cult	LQ
Onion	*Allium cepa* L.	Alliaceae	Bulb	Cult	LQ
Orange	*Citrus sinensis* (L.) Osbeck	Rutaceae	Fruit peel	Cult	HQ
Orange bitter	*Citrus aurantium* L.	Rutaceae	Fruit peel	Cult	LQ
Oregano	*Origanum* spp. *Thymbra spicata* L. *Coridothymus capitatus* Rechb. fil. *Satureja* spp. *Lippia graveolens*	Lamiaceae	Herb	Cult/wild	LQ
Palmarosa	*Cymbopogon martinii* (Roxb.) H. Wats var. *motia* Burk	Poaceae	Leaf	Cult	LQ
Parsley seed	*Petroselinum crispum* (Mill.) Nym. ex A.W. Hill	Apiaceae	Fruit	Cult	LQ

(Continued)

TABLE 3.7 (*Continued*)
Important Essential Oil-Bearing Plants—Common and Botanical Names Including Family, Plant Parts Used, Raw Material Origin, and Trade Quantities of the Essential Oil

Trade Name	Species	Plant Family	Used Plant Part(s)	Wild Collection/ Cultivation	Trade Quantities[a]
Patchouli	*Pogostemon cablin* (Blanco) Benth.	Lamiaceae	Leaf	Cult	HQ
Pennyroyal	*Mentha pulegium* L.	Lamiaceae	Herb	Cult	LQ
Pepper	*Piper nigrum* L.	Piperaceae	Fruit	Cult	LQ
Peppermint	*Mentha* x *piperita* L.	Lamiaceae	Leaf	Cult	HQ
Petitgrain	*Citrus aurantium* L. ssp. *aurantium*	Rutaceae	Leaf	Cult	LQ
Pimento leaf	*Pimenta dioica* (L.) Merr.	Myrtaceae	Fruit	Cult	LQ
Pine needle	*Pinus silvestris* L. *P. nigra* Arnold	Pinaceae	Leaf/twig	Wild	LQ
Pine needle, Dwarf	*Pinus mugo* Turra	Pinaceae	Leaf/twig	Wild	LQ
Pine silvestris	*Pinus silvestris* L.	Pinaceae	Leaf/twig	Wild	LQ
Pine white	*Pinus palustris* Mill.	Pinaceae	Leaf/twig	Wild	LQ
Rose	*Rosa* x *damascena* Miller	Rosaceae	Flower	Cult	LQ
Rosemary	*Rosmarinus officinalis* L.	Lamiaceae	Feaf	Cult/wild	LQ
Rosewood	*Aniba rosaeodora* Ducke	Lauraceae	Wood	Wild	LQ
Rue	*Ruta graveolens* L.	Rutaceae	Herb	Cult	LQ
Sage, Dalmatian	*Salvia officinalis* L.	Lamiaceae	Herb	Cult/wild	LQ
Sage, Spanish	*Salvia lavandulifolia* L.	Lamiaceae	Leaf	Cult	LQ
Sage, three lobed (Greek Turkish)	*Salvia fruticosa* Mill. (syn. *S. triloba* L.)	Lamiaceae	Herb	Cult/wild	LQ
Sandalwood, East Indian	*Santalum album* L.	Santalaceae	Wood	Wild	MQ
Sassafras, Brazilian (Ocotea cymbarum oil)	*Ocotea odorifera* (Vell.) Rohwer (*Ocotea pretiosa* [Nees] Mez.)	Lauraceae	Wood	Wild	HQ
Sassafras, Chinese	*Sassafras albidum* (Nutt.) Nees.	Lauraceae	Root bark	Wild	HQ
Savory	*Satureja hortensis* L. *Satureja montana* L.	Lamiaceae	Leaf	Cult/wild	LQ
Spearmint, Native	*Mentha spicata* L.	Lamiaceae	Leaf	Cult	MQ
Spearmint, Scotch	*Mentha gracilis* Sole	Lamiaceae	Leaf	Cult	HQ
Star anise	*Illicium verum* Hook fil.	Illiciaceae	Fruit	Cult	MQ
Styrax	*Styrax officinalis* L.	Styracaceae	Resin	Wild	LQ
Tansy	*Tanacetum vulgare* L.	Asteraceae	Flowering herb	Cult/wild	LQ
Tarragon	*Artemisia dracunculus* L.	Asteraceae	Herb	Cult	LQ
Tea tree	*Melaleuca* spp.	Myrtaceae	Leaf	Cult	LQ

(Continued)

TABLE 3.7 (*Continued*)
Important Essential Oil-Bearing Plants—Common and Botanical Names Including Family, Plant Parts Used, Raw Material Origin, and Trade Quantities of the Essential Oil

Trade Name	Species	Plant Family	Used Plant Part(s)	Wild Collection/ Cultivation	Trade Quantities[a]
Thyme	*Thymus vulgaris* L. *T. zygis* Loefl. ex L.	Lamiaceae	Herb	Cult	LQ
Valerian	*Valeriana officinalis* L.	Valerianaceae	Root	Cult	LQ
Vetiver	*Vetiveria zizanoides* (L.) Nash	Poaceae	Root	Cult	MQ
Wintergreen	*Gaultheria procumbens* L.	Ericaceae	Leaf	Wild	LQ
Wormwood	*Artemisia absinthium* L.	Asteraceae	Herb	Cult/wild	LQ
Ylang Ylang	*Cananga odorata* Hook. f. et Thoms.	Annonaceae	Flower	Cult	MQ

[a] HQ, High quantities (>1000 t/a); MQ, medium quantities (100–1000 t/a); LQ, low quantities (<100 t/a).

- Market uncertainties or political circumstances do not allow investing in long-term cultivation.
- The market is in favor of *ecologically* or *naturally* labeled wild collected material.

Especially—but not only—in developing countries, parts of the rural population depend economically on gathering high-value plant material. Less than two decades ago, almost all oregano (crude drug as well as essential oil) worldwide came from wild collection (Padulosi, 1996) and even this well-known group of species (*Origanum* sp. and *Lippia* sp.) was counted under "neglected and underutilized crops."

Yarrow (*A. millefolium s.l.*), arnica, and even chamomile originate still partly from wild collection in Central and Eastern Europe, and despite several attempts to cultivate spikenard (*Valeriana celtica*), a tiny European mountain plant with a high content of patchouli alcohol, this species is still wildly gathered in Austria and Italy (Novak et al., 1998, 2000).

To regulate the sustainable use of biodiversity by avoiding overharvesting, genetic erosion, and habitat loss, international organizations such as International Union for Conservation of Nature (IUCN), WWF/TRAFFIC, and World Health Organization (WHO) have launched together the Convention on Biological Diversity (CBD, 2001), the Global Strategy for Plant Conservation (CBD, 2002), and the Guidelines for the Sustainable Use of Biodiversity (CBD, 2004). TRAFFIC is a joint programme of World Wide Fund for Nature (WWF) and the World Conservation Union (IUCN). TRAFFIC also works in close co-operation with the Secretariat of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). These principles and recommendations address primarily the national and international policy level, but provide also the herbal industry and the collectors with specific guidance on sustainable sourcing practices (Leaman, 2006). A standard for sustainable collection and use of medicinal and aromatic plants (the international standard on sustainable wild collection of medicinal and aromatic plants [ISSC-MAP]) was issued first in 2004, and its principles will be shown at the end of this chapter. This standard certifies wild-crafted plant material insofar as conservation and sustainability are concerned. Phytochemical quality cannot, however, be derived from it, which is the reason for domestication and systematic cultivation of economically important essential oil plants.

3.5.2 Domestication and Systematic Cultivation

This offers a number of advantages over wild harvest for the production of essential oils:

- Avoidance of admixtures and adulterations by reliable botanical identification.
- Better control of the harvested volumes.
- Selection of genotypes with desirable traits, especially quality.
- Controlled influence on the history of the plant material and on postharvest handling.

TABLE 3.8
Domestication Strategy for Plants of the Spontaneous Flora

1. *Studies at the natural habitat*: botany, soil, climate, growing type, natural distribution and propagation, natural enemies, pests and diseases	→	GPS to exactly localize the place
2. *Collection of the wild grown plants and seeds*: establishment of a germplasm collection, ex situ conservation, phytochemical investigation (screening)		
3. *Plant propagation*: vegetatively or by seeds, plantlet cultivation; (biotechnol.: *in vitro* propagation)	→	Biotechnol./*in vitro*
4. *Genetic improvement*: variability, selection, breeding; phytochemical investigation, biotechnology (*in vitro* techniques)	→	Biotechnol./*in vitro*
5. *Cultivation treatments*: growing site, fertilization, crop maintenance, cultivation techniques		
6. *Phytosanitary problems*: pests, diseases	→	Biotechnol./*in vitro*
7. *Duration of the cultivation*: harvest, postharvest handling, phytochemical control of the crop produced	→	Technical processes, solar energy (new techniques)
8. *Economic evaluation and calculation*	→	New techniques

Source: Modified from Franz, Ch., 1993c. Plant Res. Dev., 37: 101; Franz, Ch., 1993d. Genetic versus climatic factors influencing essential oil formation. *Proceedings of the 12th International Congress of Essential Oils, Fragrances and Flavours*, pp. 27–44, Vienna, Austria.

On the other side, it needs arable land and investments in starting material, maintenance, and harvest techniques. On the basis of a number of successful introductions of new crops a scheme and strategy of domestication was developed by this author (Table 3.8).

Recent examples of successful domestication of essential oil-bearing plants are *oregano* (Ceylan et al., 1994; Kitiki, 1997; Putievsky et al., 1997), *Lippia* sp. (Fischer, 1998), *Hyptis suaveolens* (Grassi, 2003), and *T. lucida* (Goehler, 2006). Domesticating a new species starts with studies at the natural habitat. The most important steps are the exact botanical identification and the detailed description of the growing site. National Herbaria are in general helpful in this stage. In the course of collecting seeds and plant material, a first phytochemical screening will be necessary to recognize chemotypes (Fischer et al., 1996; Goehler et al., 1997). The phytosanitary of wild populations should also be observed so as to be informed in advance on specific pests and diseases. The flower heads of wild *Arnica montana*, for instance, are often damaged by the larvae of *Tephritis arnicae* (Fritzsche et al., 2007).

The first phase of domestication results in a germplasm collection. In the next step, the appropriate propagation method has to be developed, which might be derived partly from observations at the natural habitat: while studying wild populations of *T. lucida* in Guatemala we found, besides appropriate seed set, also runners, which could be used for vegetative propagation of selected plants (Goehler et al., 1997). Wherever possible, propagation by seeds and direct sowing is however preferred due to economic reasons.

The appropriate cultivation method depends on the plant type—annual or perennial, herb, vine, or tree—and on the agroecosystem into which the respective species should be introduced. In contrast to large-scale field production of herbal plants in temperate and Mediterranean zones, small-scale sustainable agroforesty and mixed cropping systems adapted to the environment have the preference in tropical regions (Schippmann et al., 2006). Parallel to the cultivation trials dealing with all topics from plant nutrition and maintenance to harvesting and postharvest handling, the evaluation of

the genetic resources and the genetic improvement of the plant material must be started to avoid developing of a detailed cultivation scheme with an undesired chemotype.

3.5.3 Factors Influencing the Production and Quality of Essential Oil-Bearing Plants

Since plant material is the product of a predominantly biological process, prerequisite of its productivity is the knowledge on the factors influencing it, of which the most important ones are

1. The already discussed intraspecific chemical polymorphism, derived from it the biosynthesis and inheritance of the chemical features, and as consequence selection and breeding of new cultivars.
2. The intraindividual variation between the plant parts and depending on the developmental stages ("morpho- and ontogenetic variation").
3. The modification due to environmental conditions including infection pressure and immissions.
4. Human influences by cultivation measures, for example, fertilizing, water supply, or pest management.

3.5.3.1 Genetic Variation and Plant Breeding

Phenotypic variation in essential oils was detected very early because of their striking sensorial properties. Due to the high chemical diversity, a continuous selection of the desired chemotypes leads to rather homogenous and reproducible populations, as this is the case with the landraces and common varieties. But Murray and Reitsema (1954) stated already that "a plant breeding program requires a basic knowledge of the inheritance of at least the major essential oil compounds." Such genetic studies have been performed over the last 50 years with a number of species especially of the mint family (e.g., *T. vulgaris*: Vernet, 1976; *Ocimum* sp.: Sobti et al., 1978; Gouyon and Vernet, 1982; *P. frutescens*: Koezuka et al., 1986; *Mentha* sp.: Croteau, 1991), of the Asteraceae/Compositae (*M. recutita*: Horn et al., 1988; Massoud and Franz, 1990), the genus (*Eucalyptus*: Brophy and Southwell, 2002; Doran, 2002), or the *V. zizanioides* (Akhila and Rani, 2002).

The results achieved by inheritance studies have been partly applied in targeted breeding as shown exemplarily in Table 3.9. Apart from the essential oil content and composition there are also other targets to be observed when breeding essential oil plants, as particular morphological characters ensuring high and stable yields of the respective plant part, resistances to pest and diseases as well as abiotic stress, low nutritional requirements to save production costs, appropriate homogeneity, and suitability for technological processes at harvest and postharvest, especially readiness for distillation (Pank, 2007; Bernáth, 2002). In general, the following breeding methods are commonly used (Franz, 1999).

3.5.3.1.1 Selection by Exploiting the Natural Variability

Since many essential oil-bearing species are in the transitional phase from wild plants to systematic cultivation, appropriate breeding progress can be achieved by simple selection. Wild collections or accessions of germplasm collections are the basis, and good results were obtained, for example, with *Origanum* sp. (Putievsky et al., 1997) in limited time and at low expenses.

Individual plants showing the desired phenotype will be selected and either generatively or vegetatively propagated (individual selection), or positive or negative mass selection techniques can be applied. Selection is traditionally the most common method of genetic improvement and the majority of varieties and cultivars of essential oil crops have this background. Due to the fact, however, that almost all of the respective plant species are allogamous, a recurrent selection is necessary to maintain the varietal traits, and this has especially to be considered if other varieties or wild populations of the same species are nearby and uncontrolled cross pollination may occur.

TABLE 3.9
Some Registered Cultivars of Essential Oil Plant

Species	Cultivar/ Variety	Country	Year of Registration	Breeding Method	Specific Characters
Achillea collina	SPAK	CH	1994	Crossing	High in proazulene
Angelica archangelica	VS 2	FR	1996	Recurrent pedigree	Essential oil index of roots: 180
Foeniculum vulgare	Fönicia	HU	1998	Selection	High anethole
Lavandula officinalis	Rapido	FR	1999	Polycross	High essential oil, high linalyl acetate
Levisticum officinale	Amor	PL	2000	Selection	High essential oil
Matricaria recutita	Mabamille	DE	1995	Tetraploid	High α-bisabolol
	Ciclo-1	IT	2000	Line breeding	High chamazulene
	Lutea	SK	1995	Tetraploid	High α-bisabolol
Melissa officinalis	Ildikó	HU	1998	Selection	High essential oil, Citral A + B, linalool
	Landor	CH	1994	Selection	High essential oil
	Lemona	DE	2001	Selection	High essential oil, citral
Mentha piperita	Todd's Mitcham	USA	1972	Mutation	Wilt resistant
	Kubanskaja	RUS	1980s	Crossing and polyploid	High essential oil, high menthol
Mentha spicata	MSH-20	DK	2000	Recurrent pedigree	High menthol, good flavor
Ocimum basilicum	Greco	IT	2000	Synthetic	Flavor
	Perri	ISR	1999	Cross-breeding	Fusarium Resistant
	Cardinal	ISR	2000	Cross-breeding	
Origanum syriacum	Senköy	TR	1992	Selection	5% essential oil, 60% carvacrol
	Carmeli	ISR	1999	Selection	Carvacrol
	Tavor	ISR	1999	Selection	Thymol
Origanum onites		GR	2000	Selfing	Carvacrol
Origanum hirtum		GR	2000	Selfing	Carvacrol
	Vulkan	DE	2002	Crossing	Carvacrol
	Carva	CH	2002	Crossing	Carvacrol
	Darpman	TR	1992	Selection	2.5% essential oil, 55% carvacrol
Origanum majorana	Erfo	DE	1997	Crossing	High essential oil,
(Majorana hortensis)	Tetrata	DE	1999	Ploypoid	*cis*-Sabinene-hydrate
	G 1	FR	1998	Polycross	
Salvia officinalis	Moran	ISR	1998	Crossing	Herb yield
	Syn 1	IT	2004	Synthetic	α-Thujone
Thymus vulgaris	Varico	CH	1994	Selection	Thymol/carvacrol
	T-16	DK	2000	Recurrent pedigree	Thymol
	Virginia	ISR	2000	Selection	Herb yield

The efficacy of selection has been shown by examples of many species, for instance, of the Lamiaceae family, starting from "Mitcham" peppermint and derived varieties (Lawrence, 2007), basil (Elementi et al., 2006), sage (Bezzi, 1994; Bernáth, 2000) to thyme (Rey, 1993). It is a well-known method also in the breeding of caraway (Pank et al., 1996) and fennel (Desmarest, 1992) as well as of tropical and subtropical species such as palmarosa grass (Kulkarni, 1990), tea tree (Taylor, 1996), and eucalyptus

(Doran, 2002). At perennial herbs, shrubs, and trees clone breeding, that is, the vegetative propagation of selected high-performance individual plants, is the method of choice, especially in sterile or not type-true hybrids, for example, peppermint (*M. piperita*) or lavandin (*Lavandula* × *hybrida*). But this method is often applied also at sage (Bazina et al., 2002), rosemary (Mulas et al., 2002), lemongrass (Kulkarni and Ramesh, 1992), pepper, cinnamon, and nutmeg (Nair, 1982), and many other species.

3.5.3.1.2 Breeding with Extended Variability (Combination Breeding)

If different desired characters are located in different individuals/genotypes of the same or a closely related crossable species, crossings are made followed by selection of the respective combination products. Artificial crossings are performed by transferring the paternal pollen to the stigma of the female (emasculated) or male sterile maternal flower. In the segregating progenies individuals with the desired combination will be selected and bred to constancy, as exemplarily described for fennel and marjoram by Pank (2002b).

Hybrid breeding—common in large-scale agricultural crops, for example, maize—was introduced into essential oil plants over the last decade only. The advantage of hybrids on the one side is that the F_1 generation exceeds the parent lines in performance due to hybrid vigor and uniformity ("heterosis effect") and on the other side it protects the plant breeder by segregating of the F_2 and following generations in heterogeneous low-value populations. But it needs as precondition separate (inbred) parent lines of which one has to be male sterile and one male fertile with good combining ability.

In addition, a male fertile "maintainer" line is needed to maintain the mother line. Few examples of F_1 hybrid breeding are known especially at Lamiaceae since male sterile individuals are found frequently in these species (Rey, 1994; Langbehn et al., 2002; Novak et al., 2002; Pank, 2002a).

Synthetic varieties are based on several (more than two) well-combining parental lines or clones which are grown together in a polycross scheme with open pollination for seed production. The uniformity and performance is not as high as at F_1 hybrids but the method is simpler and cheaper and the seed quality acceptable for crop production until the second or third generation. Synthetic cultivars are known for chamomile (Franz et al., 1985), arnica (Daniel and Bomme, 1991), marjoram (Franz and Novak, 1997), sage (Aiello et al., 2001), or caraway (Pank et al., 2007).

3.5.3.1.3 Breeding with Artificially Generated New Variability

Induced mutations by application of mutagenic chemicals or ionizing radiation open the possibility to find new trait expressions. Although quite often applied, such experiments are confronted with the disadvantages of undirected and incalculable results, and achieving a desired mutation is often like searching for a needle in a haystack. Nevertheless, remarkable achievements are several colchicine-induced polyploid varieties of peppermint (Murray, 1969; Lawrence, 2007), chamomile (Czabajska et al., 1978; Franz et al., 1983a ; Repčak et al., 1992), and lavender (Slavova et al., 2004).

Further possibilities to obtain mutants are studies of the somaclonal variation of *in vitro* cultures since abiotic stress in cell and tissue cultures induces also mutagenesis. Finally, genetic engineering opens new fields and potentialities to generate new variability and to introduce new traits by gene transfer. Except research on biosynthetic pathways of interesting essential oil compounds genetic engineering, GMO's and transgenic cultivars are until now without practical significance in essential oil crops and also not (yet) accepted by the consumer.

As regards the different traits, besides morphological, technological, and yield characteristics as well as quantity and composition of the essential oil, also stress resistance and resistance to pests and diseases are highly relevant targets in breeding of essential oil plants. Well known in this respect are breeding efforts against mint rust (*Puccinia menthae*) and wilt (*Verticillium dahliae*) resulting in the peppermint varieties "Multimentha," "Prilukskaja," or "Todd's Mitcham" (Murray and Todd, 1972; Lawrence, 2007; Pank, 2007), the development of *Fusarium*-wilt and *Peronospora* resistant cultivars of basil (Dudai, 2006; Minuto et al., 2006), or resistance breeding against *Septoria petroselini* in parsley and related species (Marthe and Scholze, 1996). An overview on this topic is given by Gabler (2002).

3.5.3.2 Plant Breeding and Intellectual Property Rights

Essential oil plants are biological, cultural, and technological resources. They can be found in nature gathered from the wild or developed through domestication and plant breeding. As long as the plant material is wild collected and traditionally used, it is part of the cultural heritage without any individual intellectual property and therefore not possible to protect, for example, by patents. Even finding a new plant or substance is a discovery in the "natural nature" and not an invention since a technical teaching is missing. Intellectual property, however, can be granted to new applications that involve an inventive step. Which consequences can be drawn from these facts for the development of novel essential oil plants and new selections or cultivars?

Selection and genetic improvement of aromatic plants and essential oil crops is not only time consuming but also rather expensive due to the necessity of comprehensive phytochemical and possibly molecular biological investigations. In addition, with few exceptions (e.g., mints, lavender and lavandin, parsley but also *Cymbopogon* sp., black pepper, or cloves) the acreage per species is rather limited in comparison with conventional agricultural and horticultural crops. And finally, there are several "fashion crops" with market uncertainties concerning their longevity or half-life period, respectively. The generally unfavorable cost: benefit ratio to be taken into consideration makes essential oil plant breeding economically risky and there is no incentive for plant breeders unless a sufficiently strong plant intellectual property right (IPR) exists. Questioning "which protection, which property right for which variety?" offers two options (Franz, 2001).

3.5.3.2.1 Plant Variety Protection

By conventional methods bred plant groupings that collectively are distinct from other known varieties and are uniform and stable following repeated reproduction can be protected by way of plant breeder's rights. Basis is the International Convention for the Protection of New Varieties initially issued by UPOV (Union for the Protection of New Varieties of Plants) in 1961 and changed in 1991. A plant breeder's right is a legal title granting its holder the exclusive right to produce reproductive material of his plant variety for commercial purposes and to sell this material within a particular territory for up to 30 years (trees and shrubs) or 25 years (all other plants). A further precondition is the "commercial novelty," that is, it must not have been sold commercially prior to the filing date. Distinctness, uniformity, and stability (DUS) refer to morphological (leaf shape, flower color, etc.) or physiological (winter hardiness, disease resistance, etc.), but not phytochemical characteristics, for example, essential oil content or composition. Such "value for cultivation and use (VCU) characteristics" will not be examined and are therefore not protected by plant breeder's rights (Franz, 2001; Llewelyn, 2002; Van Overwalle, 2006).

3.5.3.2.2 Patent Protection (Plant Patents)

Generally speaking, patentable are inventions (not discoveries!) that are novel, involve an innovative step, and are susceptible to industrial application, including agriculture. Plant varieties or essentially biological processes for the production of plants are explicitly excluded from patenting. But other groupings of plants that fall neither under the term "variety" nor under "natural nature" are possible to be protected by patents. This is especially important for plant groupings with novel phytochemical composition or novel application combined with an inventive step, for example, genetic modification, a technologically new production method or a novel type of isolation (product by process protection).

Especially for wild plants and essentially allogamous plants not fulfilling DUS for cultivated varieties (cultivars) and plants where the phytochemical characteristics are more important than the morphological ones, plant patents offer an interesting alternative to plant variety protection (PVP) (Table 3.10).

In conformity with the UPOV Convention of 1991 (UPOV, 1991)

- A strong plant IPR is requested.
- Chemical markers (e.g., secondary plant products) must be accepted as protectable characteristics.

TABLE 3.10
Advantages and Disadvantages of PVP versus Patent Protection of Specialist Minor Crops (Medicinal and Aromatic Plants)

PVP	Patent
Beginning of protection: registration date	Beginning of protection: application date
Restricted to "varieties"	"Varieties" not patentable, but any other grouping of plants
Requirements: DUS = distinctness, uniformity, stability	Requirements: novelty, inventive step, industrial applicability (=NIA)
Free choice of characters to be used for DUS by PVO (Plant Variety Office)	Repeatability obligatory, product by process option
Phenotypical. Mainly morphological characters (phytochemicals of minor importance)	"Essentially biological process" not patentable
Value for cultivation and use characteristics (VCU) not protected	"Natural nature" not patentable
	Claims (e.g., phytochemical characters) depend on applicant
	Phytochemical characters and use/application (VCU) patentable

- Strong depending rights for essentially derived varieties are needed since it is easy to plagiarize such crops.
- "Double protection" would be very useful (i.e., free decision by the breeder if PVR or patent protection is applied).
- But also researchers exemption and breeders privilege with fair access to genotypes for further development is necessary.

Strong protection does not hinder usage and development; it depends on a fair arrangement only (Le Buanec, 2001).

3.5.3.3 Intraindividual Variation between Plant Parts and Depending on the Developmental Stage (*Morpho-* and *Ontogenetic Variation*)

The formation of essential oils depends on the tissue differentiation (secretory cells and excretion cavities, as discussed in Section 3.3.1) and on the ontogenetic phase of the respective plant. The knowledge on these facts is necessary to harvest the correct plant parts at the right time.

Regarding the *differences between plant parts*, it is known from cinnamon (*Cinnamomum zeylanicum*) that the root-, stem-, and leaf oils differ significantly (Wijesekera et al., 1974): only the stem bark contains an essential oil with up to 70% cinnamaldehyde, whereas the oil of the root bark consists mainly of camphor and linalool, and the leaves produce oils with eugenol as main compound. In contrast to it, eugenol forms with 70%–90% the main compound in stem, leaf, and bud oils of cloves (*S. aromaticum*) (Lawrence, 1978). This was recently confirmed by Srivastava et al. (2005) for clove oils from India and Madagascar, stating in addition that eugenyl acetate was found in buds up to 8% but in leaves between traces and 1.6% only. The second main substance in leaves as well as buds is β-caryophyllene with up to 20% of the essential oil. In *Aframomum giganteum* (Zingiberaceae), the rhizome essential oil consists of β-caryophyllene, its oxide, and derivatives mainly, whereas in the leaf oil terpentine-4-ol and pinocarvone form the principal components (Agnaniet et al., 2004).

Essential oils of the Rutaceae family, especially citrus oils, are widely used as flavors and fragrances depending on the plant part and species: in lime leaves neral/geranial and nerol/geraniol are prevailing, whereas grapefruit leaf oil consists of sabinene and β-ocimene mainly. The peel of grapefruit contains almost limonene only and some myrcene, but lime peel oil shows a composition of β-pinene, γ-terpinene, and limonene (Gancel et al., 2002). In *Phellodendron* sp., Lis et al. (2004),

Lis and Milczarek (2006) found that in flower and fruit oils limonene and myrcene are dominating; in leaf oils, in contrast, α-farnesene, β-elemol, or β-ocimene, are prevailing.

Differences in the essential oil composition between the plant parts of many Umbelliferae (Apiaceae) have exhaustively been studied by the group of Kubeczka, summarized by Kubeczka et al. (1982) and Kubeczka (1997). For instance, the comparison of the essential fruit oil of aniseed (*P. anisum*) with the oils of the herb and the root revealed significant differences (Kubeczka et al., 1986). Contrary to the fruit oil consisting of almost *trans*-anethole only (95%), the essential oil of the herb contains besides anethole, considerable amounts of sesquiterpene hydrocarbons, for example, germacrene D, β-bisabolene, and α-zingiberene. Also pseudoisoeugenyl-2-methylbutyrate and epoxi-pseudoisoeugenyl-2-methylbutyrate together form almost 20% main compounds of the herb oil, but only 8.5% in the root and 1% in the fruit oil. The root essential oil is characterized by a high content of β-bisabolene, geijerene, and pregeijerene and contains only small amounts of *trans*-anethole (3.5%). Recently, Velasco-Negueruela et al. (2002) investigated the essential oil composition in the different plant parts of *Pimpinella cumbrae* from Canary Islands and found in all above-ground parts α-bisabolol as main compound besides of δ-3-carene, limonene, and others, whereas the root oil contains mainly isokessane, geijerene, isogeijerene, dihydroagarofuran, and proazulenes—the latter is also found in *Pimpinella nigra* (Kubeczka et al., 1986). Pseudoisoeugenyl esters, known as chemosystematic characters of the genus *Pimpinella*, have been detected in small concentrations in all organs except leaves.

Finally, Kurowska and Galazka (2006) compared the seed oils of root and leaf parsley cultivars marketed in Poland. Root parsley seeds contained an essential oil with high concentrations of apiole and some lower percentages of myristicin. In leaf parsley seeds, in contrast, the content of myristicin was in general higher than apiole, and a clear differentiation between flat leaved cultivars showing still higher concentrations of apiole and curled cultivars with only traces of apiole could be observed. Allyltetramethoxybenzene as the third marker was found in leaf parsley seeds up to 12.8%, in root parsley seeds, however, in traces only. Much earlier, Franz and Glasl (1976) had published already similar results on parsley seed oils comparing them with the essential oil composition of the other plant parts (Figure 3.8). Leaf oils gave almost the same fingerprint than the seeds with high myristicin in curled leaves, some apiole in flat leaves, and higher apiole concentrations than myristicin in the leaves of root varieties. In all root samples, however, apiole dominated largely over myristicin. It is therefore possible to identify the parsley type by analyzing a small seed sample.

As shown already by Figueiredo et al. (1997), in the major number of essential oil-bearing species the oil composition differs significantly between the plant parts, but there are also plant species—as mentioned before, for example, cloves—which form a rather similar oil composition in each plant organ. Detailed knowledge in this matter is needed to decide, for instance, how exact the separation of plant parts has to be performed before further processing (e.g., distillation) or use.

Another topic to be taken into consideration is the *developmental stage* of the plant and the plant organs, since the formation of essential oils is phase dependent. In most cases, there is a significant increase of the essential oil production throughout the whole vegetative development.

And especially in the generative phase between flower bud formation and full flowering, or until fruit or seed setting, remarkable changes in the oil yield and compositions can be observed. Obviously, a strong correlation is given between formation of secretory structures (oil glands, ducts, etc.) and essential oil biosynthesis, and different maturation stages, are associated with, for example, higher rates of cyclization or increase of oxygenated compounds (Figueiredo et al., 1997).

Investigations on the ontogenesis of fennel (*F. vulgare* Mill.) revealed that the best time for picking fennel seeds is the phase of full ripeness due to the fact that the anethole content increases from <50% in unripe seeds to over 80% in full maturity (Marotti et al., 1994). In dill weed (*Anethum graveolens* L.) the content on essential oil rises from 0.1% only in young sprouts to more than 1% in herb with milk ripe umbels (Gora et al., 2002). In the herb, oil α-phellandrene prevails until the beginning of flowering with up to 50%, followed by dill ether, *p*-cymene, and limonene. The oil from green as well as ripe umbels contains, on the other hand, mainly (*S*)-carvone and (*R*)-limonene. The

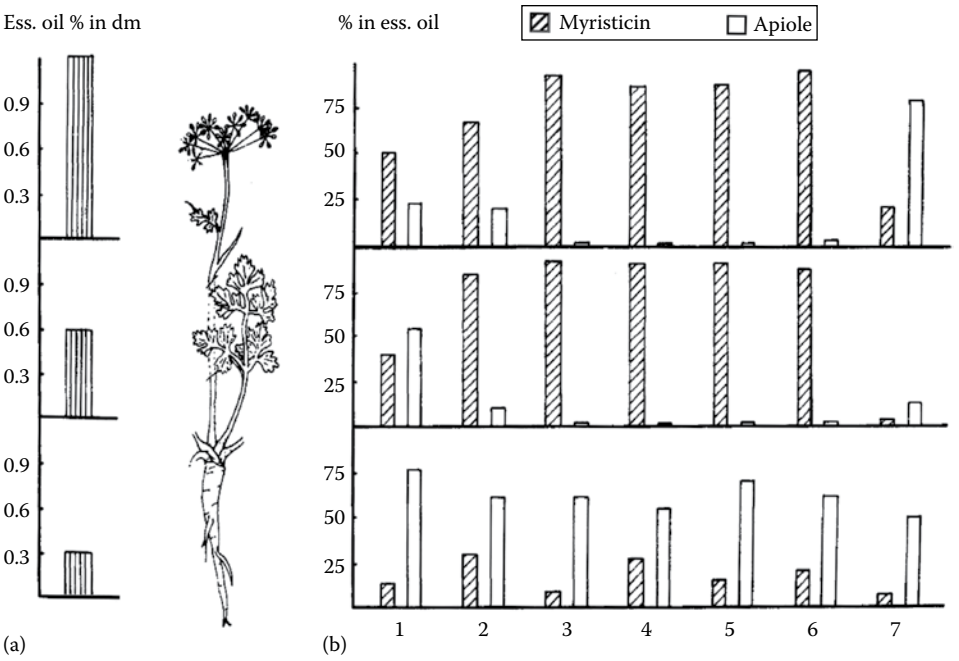

FIGURE 3.8 Differences in the essential oil of fruits, leaves, and roots of parsley cultivars (*Petroselinum crispum* (Mill.) Nyman). (a) Essential oil content, (b) content of myristicin and apiole in the essential oil. 1, 2—flat leaved cv's; 3–7—curled leaves cv's; 7—root parsley).

flavor of dill oil changes therefore dramatically, which has to be considered when determining the harvest time for distillation.

Among Compositae (Asteraceae) there are not as many results concerning ontogeny due to the fact that in general the flowers or flowering parts of the plants are harvested, for example, chamomile (*M. recutita*), yarrow (*A. millefolium s.l.*), immortelle (*Helichrysum italicum*), or wormwood (*Artemisia* sp.) and therefore the short period between the beginning of flowering and the decay of the flowers is of interest only. In chamomile (*M. recutita*), the flower buds show a relatively high content on essential oil between 0.8% and 1.0%, but the oil yield in this stage is rather low. From the beginning of flowering, the oil content increases until full flowering (all disc florets open) and decreases again with decay of the flower heads. At full bloom there is also the peak of (pro) chamazulene, whereas farnesene and α-bisabolol decrease from the beginning of flowering and the bisabololoxides rise (Franz et al., 1978). This was confirmed by Repčak et al. (1980). The essential oil of *Tagetes minuta* L. at different development stages was investigated by Worku and Bertoldi (1996). Before flower bud formation the oil content was 0.45% only, but it culminated with 1.34% at the immature seed stage. During this period *cis*-ocimene increased from 7.2% to 37.5% and *cis*-ocimenone declined from almost 40%–13.1%. Little variations could be observed at *cis*- and *trans*-tagetone only. Similar results have been reported also by Chalchat et al. (1995).

Also for *Lippia* sp. (Verbenaceae) some results are known concerning development stages (Fischer, 1998; Coronel et al., 2006). The oil content in the aerial parts increases from young buds (<1.0%) to fully blooming (almost 2.0%). But although quantitative variations could be observed for most components of the essential oils, the qualitative composition appeared to be constant throughout the growing season.

A particular situation is given with eucalypts as they develop up to five distinct types of leaves during their lifetime, each corresponding to a certain ontogenetic stage with changing oil concentrations and compositions (Doran, 2002). Usually the oil content increases from young to

matured, nonlignified leaves, and is thereafter declining until leaf lignification. Almost the same curve is valid also for the 1,8-cineole concentration in the oil. But comparing the relatively extensive literature on this topic, one may conclude that the concentration at various stages of leaf maturity is determined by a complex pattern of quantitative change in individual or groups of substances, some remaining constant, some increasing, and some decreasing. Tsiri et al. (2003) investigated the volatiles of the leaves of *Eucalyptus camaldulensis* over the course of a year in Greece and found a seasonal variation of the oil concentration with a peak during summer and lowest yields during winter. The constituent with highest concentration was 1,8-cineole (25.3%–44.2%) regardless the time of harvest. The great variation of all oil compounds showed however no clear tendency, neither seasonal nor regarding leaf age or leaf position. Doran (2002) concluded therefore that genotypic differences outweigh any seasonal or environmental effects in eucalypts.

There is an extensive literature on ontogenesis and seasonal variation of Labiatae essential oils. Especially for this plant family, great differences are reported on the essential oil content and composition of young and mature leaves and the flowers may in addition influence the oil quality significantly. Usually, young leaves show higher essential oil contents per area unit compared to old leaves. But the highest oil yield is reached at the flowering period, which is the reason that most of the oils are produced from flowering plants. According to Werker et al. (1993) young basil (*O. basilicum*) leaves contained 0.55% essential oil while the content of mature leaves was only 0.13%. The same is also valid to a smaller extent for *O. sanctum*, where the essential oil decreases from young (0.54%) to senescing leaves (0.38%) (Dey and Choudhuri, 1983). Testing a number of basil cultivars mainly of the linalool chemotype, Macchia et al. (2006) found that only some of the cultivars produce methyl eugenol up to 8% in the vegetative stage. Linalool as main compound is increasing from the vegetative (10%–50%) to the flowering (20%–60%), and postflowering phase (25%–80%), whereas the second important substance eugenol reaches its peak at the beginning of flowering (5%–35%). According to the cultivars, different harvest dates are therefore recommended. In *O. sanctum*, the content of eugenol (60.3%–52.2%) as well as of methyl eugenol (6.6%–2.0%) is decreasing from young to senescent leaves and at the same time β-caryophyllene increases from 20.8% to 30.2% (Dey and Choudhuri, 1983).

As regards oregano (*O. vulgare* ssp. *hirtum*), the early season preponderance of *p*-cymene over carvacrol was reversed as the season progressed and this pattern could also be observed at any time within the plant, from the latest leaves produced (low in cymene) to the earliest (high in cymene) (Johnson et al., 2004; Figure 3.9). Already Kokkini et al. (1996) had shown that oregano contains a higher proportion of *p*-cymene to carvacrol (or thymol) in spring and autumn, whereas carvacrol/thymol prevails in the summer. This is explained by Dudai et al. (1992) as photoperiodic reaction: short days with high *p*-cymene, long days with low *p*-cymene production. But only young plants are

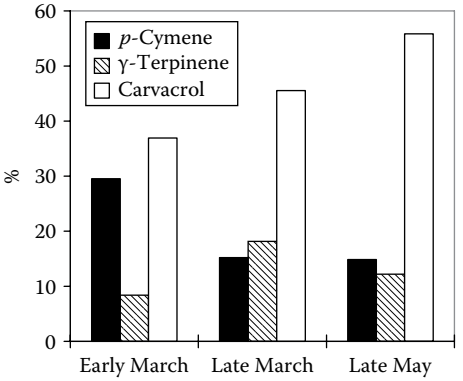

FIGURE 3.9 Average percentages and concentrations of *p*-cymene. γ-Terpinene and carvacrol at the different sampling dates of *Origanum vulgare ssp. hirtum*.

capable of making this switch, whereas in older leaves the already produced and stored oil remains almost unchanged (Johnson et al., 2004).

Presumably the most studied essential oil plant is peppermint (*M. piperita* L.). Already in the 1950s Lemli (1955) stated that the proportion of menthol to menthone in peppermint leaves changes in the course of the development toward higher menthol contents. Lawrence (2007) has just recently shown that from immature plants via mature to senescent plants the content of menthol increases (34.8%–39.9%–48.2%) and correspondingly the menthone content decreases dramatically (26.8%–17.4%–4.7%). At the same time, also an increase of menthyl acetate from 8.5% to 23.3% of the oil could be observed. At full flowering, the peppermint herb oil contains only 36.8% menthol but 21.8% menthone, 7.7% menthofuran, and almost 3% pulegone due to the fact that the flower oils are richer in menthone and pulegone and contain a high amount of menthofuran (Hefendehl, 1962). Corresponding differences have been found between young leaves rich in menthone and old leaves with high menthol and menthyl acetate content (Hoeltzel, 1964; Franz, 1972). The developmental stage depends, however, to a large extent from the environmental conditions, especially the day length.

3.5.3.4 Environmental Influences

Essential oil formation in the plants is highly dependent on climatic conditions, especially day length, irradiance, temperature, and water supply. Tropical species follow in their vegetation cycle the dry and rainy season; species of the temperate zones react more on day length, the more distant from the equator their natural distribution area is located.

Peppermint as typical long day plant needs a minimum day length (hours of day light) to switch from the vegetative to the generative phase. This is followed by a change in the essential oil composition from menthone to menthol and menthyl acetate (Hoeltzel, 1964). Franz (1981) tested six peppermint clones at Munich/Germany and at the same time also at Izmir/Turkey. At the development stage "beginning of flowering," all clones contained at the more northern site much more menthol than on the Mediterranean location, which was explained by a maximum day length in Munich of 16 h 45 min, but in Izmir of 14 h 50 min only. Comparable day length reactions have been mentioned already for oregano (Dudai et al., 1992; Kokkini et al., 1996). Also marjoram (*O. majorana* L.) was influenced not only in flower formation by day length, but also in oil composition (Circella et al., 1995). At long day treatment the essential oil contained more *cis*-sabinene hydrate. Terpinene-4-ol prevailed under short day conditions.

Franz et al. (1986) performed ecological experiments with chamomile, growing vegetatively propagated plants at three different sites, in South Finland, Middle Europe, and West Turkey. As regards the oil content, a correlation between flower formation, flowering period, and essential oil synthesis could be observed: the shorter the flowering phase, the less was the time available for oil formation, and thus the lower was the oil content. The composition of the essential oil, on the other hand, showed no qualitative change due to ecological or climatic factors confirming that chemotypes keep their typical pattern. In addition, Massoud and Franz (1990) investigated the genotype–environment interaction of a chamazulene–bisabolol chemotype. The frequency distributions of the essential oil content as well as the content on chamazulene and α-bisabolol have shown that the highest oil- and bisabolol content was reached in Egypt while under German climatic conditions chamazulene was higher. Similar results have been obtained by Letchamo and Marquard (1993). The relatively high heritability coefficients calculated for some essential oil components—informing whether a character is more influenced by genetic or other factors—confirm that the potential to produce a certain chemical pattern is genetically coded, but the gene expression will be induced or repressed by environmental factors also (Franz, 1993b,d).

Other environmental factors, for instance, soil properties, water stress, or temperature, are mainly influencing the productivity of the respective plant species and by this means the oil yield also, but have little effect on the essential oil formation and composition only (Figueiredo et al., 1997; Salamon, 2007).

3.5.3.5 Cultivation Measures, Contaminations, and Harvesting

Essential oil-bearing plants comprise annual, biennial, or perennial herbs, shrubs, and trees, cultivated either in tropical or subtropical areas, in Mediterranean regions, in temperate, or even in arid zones. Surveys in this respect are given, for instance, by Chatterjee (2002) for India, by Carruba et al. (2002) for Mediterranean environments, and by Galambosi and Dragland (2002) for Nordic countries. Nevertheless, some examples should refer to some specific items.

The *cultivation method*—if direct sowing or transplanting—and the timing influence the crop development and by that way also the quality of the product, as mentioned above. Vegetative propagation, necessary for peppermint due to its genetic background as interpecific hybrid, common in *Cymbopogon* sp. and useful to control the ratio between male and female trees in nutmeg (*Myristica fragrans*), results in homogeneous plant populations and fields. A disadvantage could be the easier dispersion of pests and diseases, as known for "yellow rot" of lavandin (*Lavandula* × *hybrida*) (Fritzsche et al., 2007). Clonal propagation can be performed by leaf or stem cuttings (Goehler et al., 1997; El-Keltawi and Abdel-Rahman, 2006; Nicola et al., 2006) or *in vitro* (e.g., Figueiredo et al., 1997; Mendes and Romano, 1997), the latter method especially for mother plant propagation due to the high costs. *In vitro* essential oil production received increased attention in physiological experiments, but has up to now no practical significance.

As regards *plant nutrition and fertilizing*, a numerous publications have shown its importance for plant growth, development, and biomass yield. The essential oil yield, obviously, depends on the plant biomass; the oil percentage is partly influenced by the plant vigor and metabolic activity. Optimal fertilizing and water supply results in better growth and oil content, for example, in marjoram, oregano, basil, or coriander (Menary, 1994), but also in delay of maturity, which causes quite often "immature" flavors.

Franz (1972) investigated the influence of nitrogen and potassium on the essential oil formation of peppermint. He could show that higher nitrogen supply increased the biomass but retarded the plant development until flowering, whereas higher potassium supply forced the maturity. With increasing nitrogen, a higher oil percentage was observed with lower menthol and higher menthone content; potassium supply resulted in less oil with more menthol and menthyl acetate. Comparable results with *R. officinalis* have been obtained by Martinetti et al. (2006), and Omidbaigi and Arjmandi (2002) have shown for *T. vulgaris* that nitrogen and phosphorus fertilization had significant effect on the herb yield and essential oil content, but did not change the thymol percentage. Also Java citronella (*Cymbopogon winterianus* Jowitt.) responded to nitrogen supply with higher herb and oil yields, but no influence on the geraniol content could be found (Munsi and Mukherjee, 1986).

Extensive pot experiments with chamomile (*M. recutita*) have also shown that high nitrogen and phosphorus nutrition levels resulted in a slightly increased essential oil content of the anthodia, but raising the potassium doses had a respective negative effect (Franz et al., 1983a,b). With nitrogen the flower formation was in delay and lasted longer; with more potassium the flowering phase was reduced, which obviously influenced the period available for essential oil production. This was confirmed by respective ^{14}C-acetate labeling experiments (Franz, 1981).

Almost no effect has been observed on the composition of the essential oil. Also a number of similar pot or field trials came to the same result, as summarized by Salamon (2007).

Salinity and salt stress get an increasing importance in agriculture especially in subtropical and Mediterranean areas. Some essential oil plants, for example, *Artemisia* sp. and *M. recutita* (chamomile) are relatively salt tolerant. Also thyme (*T. vulgaris*) showed a good tolerance to irrigation water salinity up to 2000 ppm, but exceeding concentrations caused severe damages (Massoud et al., 2002). Higher salinity reduced also the oil content, and an increase of *p*-cymene was observed. Recently, Aziz et al. (2008) investigated the influence of salt stress on growth and essential oil in several mint species. In all three mints, salinity reduced the growth severely from 1.5 g/L onward; in peppermint, the menthone content raised and menthol went down to $<$1.0%, in apple mint, linalool and neryl acetate decreased while myrcene, linalyl acetate, and linalyl propionate increased.

Further problems to be taken into consideration in plant production are *contaminations* with heavy metals, damages caused by pests and diseases, and *residues* of plant protection products. The

most important toxic heavy metals Cd, Hg, Pb, and Zn, but also Cu, Ni, and Mn may influence the plant growth severely and by that way also the essential oil, as they may act as cofactors in the plant enzyme system. But as contaminants, they remain in the plant residue after distillation (Zheljazkov and Nielsen, 1996; Zheljazkov et al., 1997). Some plant species, for example, yarrow and chamomile accumulate heavy metals to a greater extent. This is, however, problematic for using the crude drug or for deposition of distillation wastes mainly. The same is valid for the microbial contamination of the plant material. More important in the production of essential oils are pests and diseases that cause damages to the plant material and sometimes alterations in the biosynthesis; but little is known in this respect.

In contrast to organic production, where no use of pesticides is permitted, a small number of insecticides, fungicides, and herbicides are approved for conventional herb production. The number, however, is very restricted (end of 2008 several active substances lost registration at least in Europe), and limits for residues can be found in national law and international regulations, for example, the European Pharmacopoeia. For essential oils, mainly the lipophilic substances are of relevance since they can be enriched over the limits in the oil.

Harvesting and the first steps of *postharvest handling* are the last part of the production chain of starting materials for essential oils. The harvest date is determined by the development stage or maturity of the plant or plant part, Harvesting techniques should keep the quality by avoiding adulterations, admixtures with undesired plant parts, or contaminations, which could cause "off-flavor" in the final product. There are many technical aids at disposal, from simple devices to large-scale harvesters, which will be considered carefully in Chapter 4. From the quality point of view, raising the temperature by fermentation should in general be avoided (except, in vanilla), and during the drying process further contamination with soil, dust, insects, or molds has to be avoided.

Quality and safety of essential oil-bearing plants as raw materials for pharmaceutical products, flavors, and fragrances are of highest priority from the consumer point of view. To meet the respective demands, standards and safety as well as quality assurance measures are needed to ensure that the plants are produced with care, so that negative impacts during wild collection, cultivation, processing, and storage can be limited. To overcome these problems and to guarantee a steady, affordable and

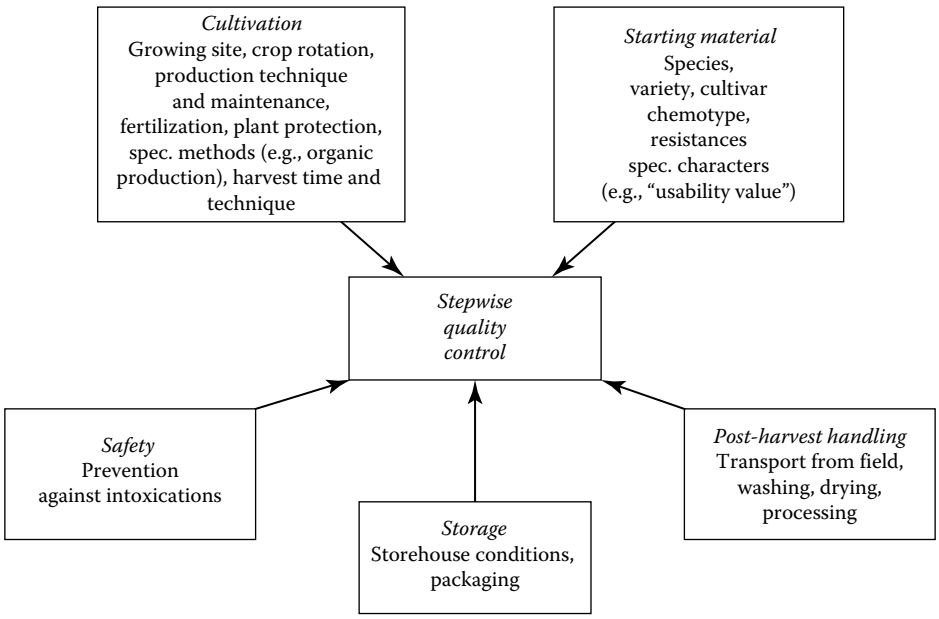

FIGURE 3.10 Main items of "good agricultural practices" (GAP) for medicinal and aromatic plants.

sustainable supply of essential oil plants of good quality (Figure 3.10), in recent years guidelines for good agricultural practices (GAP) and standards for Sustainable Wild Collection (ISSC) have been established at the national and international level.

3.6 INTERNATIONAL STANDARDS FOR WILD COLLECTION AND CULTIVATION

3.6.1 GA(C)P: Guidelines for Good Agricultural (and Collection) Practice of Medicinal and Aromatic Plants

First initiatives for the elaboration of such guidelines trace back to a roundtable discussion in Angers, France in 1983, and intensified at an International Symposium in Novi Sad 1988 (Franz, 1989b). A first comprehensive paper was published by Pank et al. (1991) and in 1998 the European Herb Growers Association (EHGA/EUROPAM) released the first version (Máthé and Franz, 1999). The actual version can be downloaded from http://www.europam.net.

In the following it was adopted and slightly modified by the European Agency for the Evaluation of Medicinal Products (EMEA), and finally as Guidelines on good agricultural and collection practices (GACP) by the WHO in 2003.

All these guidelines follow almost the same concept dealing with the following topics:

- Identification and authentication of the plant material, especially botanical identity and deposition of specimens.
- Seeds and other propagation material, respecting the specific standards and certifications.
- Cultivation, including site selection, climate, soil, fertilization, irrigation, crop maintenance, and plant protection with special regard to contaminations and residues.
- Harvest, with specific attention to harvest time and conditions, equipment, damage, contaminations with (toxic) weeds and soil, transport, possible contact with any animals, and cleaning of all equipment and containers.
- Primary processing, that is, washing, drying, distilling; cleanness of the buildings; according to the actual legal situation these processing steps including distillation—if performed by the farmer—is still part of GA(C)P; in all other cases, it is subjected to GMP (good manufacturing practice).
- Packaging and labeling, including suitability of the material.
- Storage and transportation, especially storage conditions, protection against pests and animals, fumigation, and transport facilities.
- Equipment: material, design, construction, easy to clean.
- Personnel and facilities, with special regard to education, hygiene, protection against allergens and other toxic compounds, welfare.

In the case of wild collection the standard for sustainable collection should be applied (see Section 3.3.6.2).

A very important topic is finally the *documentation* of all steps and measurements to be able to trace back the starting material, the exact location of the field, any treatment with agrochemicals, and the special circumstances during the cultivation period. Quality assurance is only possible if the traceability is given and the personnel is educated appropriately. Certification and auditing of the production of essential oil-bearing plants is not yet obligatory, but recommended and often requested by the customer.

3.6.2 ISSC-MAP: The International Standard on Sustainable Wild Collection of Medicinal and Aromatic Plants

ISSC-MAP is a joint initiative of the German Bundesamt für Naturschutz (BfN), WWF/TRAFFIC Germany, IUCN Canada, and IUCN Medicinal Plant Specialist Group (MPSG). ISSC-MAP intends to ensure the long-term survival of MAP populations in their habitats by setting principles and

criteria for the management of MAP wild collection (Leaman, 2006; Medicinal Plant Specialist Group, 2007). The standard is not intended to address product storage, transport, and processing, or any issues of products, topics covered by the WHO Guidelines on GACP for Medicinal Plants (WHO, 2003). ISSC-MAP includes legal and ethical requirements (legitimacy, customary rights, and transparency), resource assessment, management planning and monitoring, responsible collection, and collection, area practices and responsible business practices. One of the strengths of this standard is that resource management not only includes target MAP resources and their habitats but also social, cultural, and economic issues.

3.6.3 FairWild

The FairWild standard (http://www.fairwild.org) was initiated by the Swiss Import Promotion Organization (SIPPO) and combines principles of FairTrade (Fairtrade Labelling Organizations International, FLO), international labor standards (International Labour Organization, ILO), and sustainability (ISSC-MAP).

3.7 CONCLUSION

This chapter has shown that a number of items concerning the plant raw material have to be taken into consideration when producing essential oils. A quality management has to be established tracing back to the authenticity of the starting material and ensuring that all known influences on the quality are taken into account and documented in an appropriate way. This is necessary to meet the increasing requirements of international standards and regulations. The review also shows that a high number of data and information exist, but sometimes without expected relevance due to the fact that the repeatability of the results is not given by a weak experimental design, an incorrect description of the plant material used, or an inappropriate sampling. On the other side, this opens the chance for many more research work in the field of essential oil-bearing plants.

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4 Natural Variability of Essential Oil Components

Éva Németh-Zámbori

CONTENTS

4.1 MANIFESTATION OF VARIABILITY

It is a long known fact that qualitative and quantitative composition of genuine essential oils is not a standard one. In consequence of this, they possess different quality, value, and price on the market.

As a reflection of this practical experience, in several cases, different qualities are defined for essential oils of the same species. In the International Organization for Standardization (ISO) standard series (ISO TC/54), numerous essential oils are listed in at least two (e.g., lemongrass, thyme)—but in some cases even in four (e.g., petitgrain, spearmint)—different qualities depending on the geographical source, plant organ, or main component of the oil (ISO, 2013). However, numerous other factors might contribute to the different qualities, such as variety, environment, agricultural methods, or extraction technology. In practice, the same species might be utilized for different applications based on the variable composition of its oil, like the thyme-odor type, lavender-odor type, and rose-odor type individuals of *Thymus longicaulis* subsp. *longicaulis* (Baser et al., 1993).

In several cases, the real sources of variability are hard to determine. However, for standardization of any product, it is of primary importance that the background of variability and the factors, which influence the composition of the essential oils, are detected and can be managed and controlled.

In the scientific literature, reports on variability of essential oil components are very frequently published. According to a survey on articles in the last volumes (2010–2018) of *Journal of Essential Oil Research* (Taylor & Francis Group), it can be established that more than one-third of them is evaluating biological variability at specific or intraspecific taxonomic levels or chemosyndromes due to developmental or morphological differences (Figure 4.1).

The chemical variability of the essential oils gained from different plant species varies on a large scale. Tétényi (1975) mentioned already fifty years ago that 36 families, 121 genera, 360 species are polymorphs for essential oils. This number must have increased enormously since that time because of intensive research and highly developed analytical techniques.

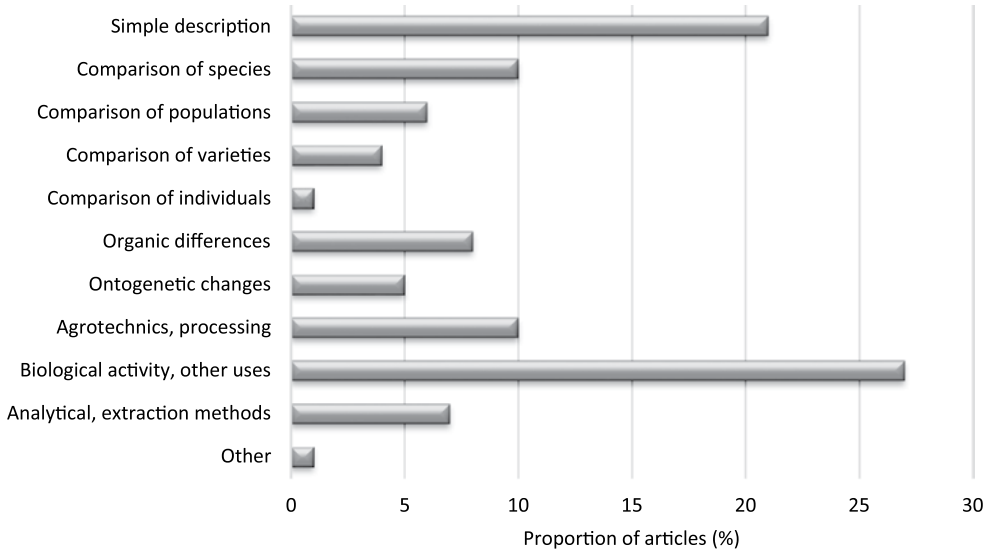

FIGURE 4.1 Distribution of topics of publications in *Journal of Essential Oil Research* between 2010 and 2018.

The backgrounds of the chemical variability of essential oil composition are usually grouped as abiotic and biotic factors. Abiotic influencing factors include the effects of the environment (exposure, soil, light intensity and length of illumination, wind, absolute and marginal temperatures, water supply as total, and frequency of precipitation) but also those in consequence of human activities (agrotechnical methods, extraction, processing, and storage). Several chapters of this book deal with these factors in detail.

The biotic/biological factors are the main topic of this chapter. Natural variability may be defined as the phenomenon when a diverse quality of essential oils is detectable as result of genetic-biological differences of the source plants. "Natural variability" is, however, a rather complex issue having many aspects, as we can see below. In this context, we deal with the essential oil spectrum, quantitative and qualitative composition of the oils, and do not discuss other chemical or physical properties of the oils. We also have to declare that although based on the accepted definition, essential oils are products produced from the plant by physical means like distillation or pressing; in this chapter, in some cases, we refer to "essential oil" also as the mixture of volatile compounds in the plant *in vivo.*

4.2 VARIABILITY AT DIFFERENT TAXONOMIC LEVELS

4.2.1 Species

Variability in the composition of essential oils has been most frequently discussed at the level of plant species and has the highest relevance from practical points of view.

A significant variation in qualitative and quantitative composition might have considerable influence on the recognition and the market value both of the drug and the essential oil itself. Besides, fluctuations in the composition of the essential oil might have significant effects on the therapeutic efficacy or sensory value of the product. Limonene seems to have a strong influence on the allelopathic property of *Tagetes minuta* (Scrivanti et al., 2003). The characteristic antioxidative property of thyme (*Thymus vulgaris*) oil is by 2.0–2.6 times higher in chemotypes containing phenolic compounds as main components (Chizzola et al., 2008). In some phytotherapeutic preparations of chamomile, the antiphlogistic and spasmolytic effect seems to be in closest connection with the content of (−)-α-bisabolol (Schilcher, 2004). Recently, it has also been demonstrated that individual

compounds may be identified as putative biomarkers to the active oils (Maree et al., 2014; Ayouniet al., 2016). Anti-quorum-sensing activity of certain essential oils may be attributed to the presence of special compounds like eugenol, geraniol geranial, menthol, and pulegone where, nevertheless, synergistic effects are of high importance (Mokhetho et al., 2018). On the other hand, adverse effects may be caused by the presence of single compounds like the carcinogen effect of *cis*-isoasarone in the essential oil of calamus (*Acorus calamus*), (Blaschek et al., 1998) or the high concentrations of thujone in wormwood (*Artemisia absinthium*) or sage (*Salvia officinalis*) oils (Lachenmeier et al., 2006).

Not each species exhibits a similar amount of variability. A huge amount of research data accumulated in the last decades proving that the incidence of diversity is one of the characteristic features of the plant species.

The well-known caraway (*Carum carvi*) seems to be an essential oil–bearing species of relatively low variability concerning the oil constituents. Nowadays, besides being a popular spice, it is a source of essential oil of excellent antimicrobial properties, but the spasmolytic and cholagogue effects justify its use in phytotherapy, too. In the oil of caraway, the ratio of the main components *S*(+) carvone and *R*(+) limonene in the oil is above 90%, most frequently above 95% (Table 4.1). Variability is manifested in most cases only in their proportions compared to each other. Minor constituents have been rarely identified and mentioned. The majority of constituents are all monoterpenes, besides the sesquiterpene β-caryophyllene and some phenolic and aliphatic compounds.

Biological variability of the oil composition seems to be more pronounced if comparing the two varieties (*Carum carvi* var. *annuum* and var. *biennis*) of caraway. In general, biennial varieties are believed to accumulate higher concentrations of total volatiles and carvone (Table 4.1). Bouwmeester and Kuijpers (1993) concluded that the restricted potential of carvone accumulation in annual varieties is the consequence of limited availability of assimilates. Indeed, the abundance of nutrients available from the more robust root system of biennial varieties might play a very important role in accumulation of secondary compounds, as the examples of other species also show (Bodor et al., 2006, 2009).

TABLE 4.1
Variability of the Main Components Carvone and Limonene in Biennial and Annual Accessions of Caraway

	Biennial		Annual	
Source of Data	**Carvone**	**Limonene**	**Carvone**	**Limonene**
Aćimović et al. (2014)	–	–	27–44	54–70
Argañosa et al. (1998)	54–57	43–45	46–50	49–53
Embong et al. (1977)	39–46	43–49	–	–
Fleischer and Fleischer (1988)	54–68	30–44	–	–
Forwick-Kreutzer et al. (2003)	52–72	–	–	–
Galambosi and Peura (1996)	47–49	39–52	–	–
Laribi et al. (2010)	–	–	76–80	13–20
Pank et al. (2008)	–	–	50–53	45–48
Puschmann et al. (1992)	47–54	–	45–52	–
Putievsky et al. (1994)	53–59	38–44	47–62	3–46
Raal et al. (2012)	44–95[a]	2–50	–	–
Sedláková et al. (2003)	72–81	18–27	–	–
Solberg et al. (2016)	14–15	69–71	–	–
Zámboriné (2005)	51–60	38–44	50–56	43–49

[a] Annual accessions might also be included.

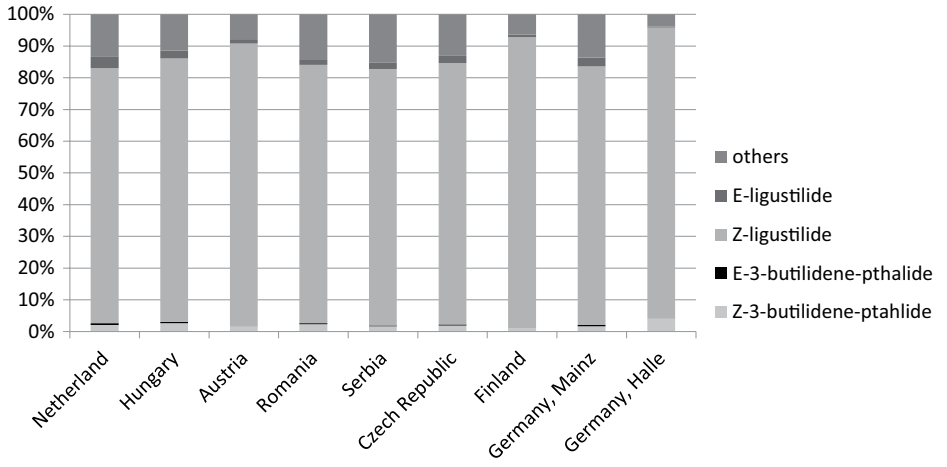

FIGURE 4.2 Distribution of characteristic phthalide components (in %) in the root oil of different European accessions of lovage (*Levisticum officinale*). (Adapted from Németh, É. et al., Unpublished.)

Based on the majority of available references, the carvone content of biennial accessions is regularly higher than the content of the annual plants. This fact may explain why biennial caraway is still in many countries in cultivation although production of the annual variety has an economic advantage based on higher seed yields and more advantageous crop rotation.

In Apiaceae species, a relatively low biological variability in essential oil composition is usual. Another example may be lovage (*Levisticum officinale*), whose aromatic volatile (essential oil) and non-volatile (mainly coumarin-type) compounds justify the application of each part of the plant as a popular spice. However, the most valuable organ is the root. The main components of the root essential oil are alkyl-phthalide type compounds, among which the most abundant ones are usually Z-ligustilide and butylidene-phthalide (Szebeni et al., 1992; Venskutonis, 1995; Novák, 2006). Only in exceptional cases have other compounds been detected as major ones, like 49% phellandrene (Scottish accession) or 26% terpinyl acetate (Dutch accession) in the investigations of Raal et al. (2008). Our own investigations on ten accessions of lovage originating from different European countries ascertained that the compositional variability is low (unpublished). The phthalides are the main components of the distilled oil practically in each accession (Figure 4.2). The presence of two isomers, E and Z, makes the pictures somewhat more diverse; however, their ratios are not significantly different in either of the accessions. In each case, the Z isomer is in multiple concentrations present more than the other one.

The seeds of the investigated accessions have been obtained from different countries and regions but—as in many cases—the real genetic origin is uncertain. Therefore, a common basic source cannot be excluded, either. However, even in this case, it might mean that lovage has a very narrow gene pool and maybe therefore possess a low chemical variability. The connection between the restricted natural distribution and small spectral variance of the oil might support the hypothesis on the development of polychemism as a tool in geographical distribution and ecological adaptation (see Section 4.5).

The Mediterranean species hyssop (*Hyssopus officinalis*) belongs to the Lamiaceae family. It is used for its spicy essential oil in the food industry and also as a strong antimicrobial agent.

Monoterpenes, which are present as the main compounds in the oil of this species (pinocamphone, isopinocamphone), are relatively seldom detected in higher quantities in essential oils of other species. Although, as the highest number of 44 components were detected in hyssop oil (Chalchat et al., 2001), the major ones are relatively uniform and found almost in each examined accession (Table 4.2). Besides the mentioned compounds, the majority of further ones are also monoterpenes,

TABLE 4.2
Main Components in the Essential Oil of Hyssop (*Hyssopus Officinalis*) According to Different References

Main Compounds (In the Row of Their Abundance)	Reference
Pinocamphone, isopinocamphone, β-pinene	Aiello et al. (2001)
Pinocarvone, isopinocamphone, β-pinene	Bernotienė and Butkienė (2010)
Pinocamphone, isopinocamphone	Chalchat et al. (2001)
Isopinocamphone, β-pinene	Danila et al. (2012)
Pinocamphone, isopinocamphone, germacrene D, pinocarvone	Galambosi et al. (1993)
Pinocamphone, isopinocamphone, β-pinene	Fraternale et al. (2004)
Terpineol, bornyl acetate, linalool	Hodzsimatov and Ramazanova (1974)
Isopinocamphone, β-pinene, pinocamphone	Joulain and Ragault (1976)
Isopinocamphone, β-pinene, pinocarvone	Kizil et al. (2010)
Isopinocamphone, pinocamphone, β-pinene	Koller and Range (1997)
Pinocamphone, β-pinene	Lawrence (1979)
Pinocamphone, isopinocamphone, β-pinene, pinocarvone	Lawrence (1992)
Isopinocamphone, pinocamphone	Mitič and Đorđević (2000)
Isopinocamphone, pinocamphone, β-pinene	Németh-Zámbori et al. (2017)
Isopinocamphone, myrtenol, β-pinene, 1,8-cineole, methyl-eugenol, limonene	Piccaglia et al. (1999)
Pinocamphone, camphor, β-pinene	Schulz and Stahl-Biskup (1991)
Isopinocamphone, 1,8-cineole, β-pinene	Tsankova et al. (1993)
1,8-Cineole, β-pinene	Vallejo et al. (1995)

products of related biosynthetic pathways (β-pinene, pinocarvone, and myrtenol). In general, it can be observed that besides some mentioned main compounds, all the others are present only in minimal concentrations (Németh-Zámbori et al., 2017). Thus, the biological variability of the herb oil of hyssop is relatively low. Only samples of the subspecies *aristatus* (Godr.) Briq., collected from three populations of Appennines, showed a different character with higher amounts of myrtenol (up to 32%), methyl-eugenol (up to 44%) and limonene (up to 15%); however, the characteristic pinane-type compounds have also been found here at different quantities (Piccaglia et al., 1999).

Summarizing the above-mentioned species, it seems to be clear that the variation in the oil composition of the above-mentioned species is principally a quantitative one. The spectrum seems to be relatively constant; changes are detectable basically in the accumulation proportions of the individual components.

On the other side, a great number of plant species can be characterized by high intraspecific variability concerning essential oil composition. In these oils, both qualitative and quantitative variations are present.

One of the most comprehensively studied genera from this respect is the genus *Achillea*. For the majority of yarrow species, a wide variability in oil composition has been detected. Based on a comprehensive literature search, in most of the species, one to three compounds have been identified as main components (Kindlovits and Németh, 2012). The evaluation is, however, not a simple one because, in most cases, the different chemical races had been detected and mentioned by different authors independently from each other. Therefore, the comparison of data is always a hard task taking into account the possible role of other influencing factors besides the genetic background.

Chamazulene is currently the most important component of the distilled oil of yarrow. In general, the proazulene accumulation potential of *A. collina* (4*n*) and its relatives *A. asplenifolia* and *A. roseo-alba* (2*n*) seems to be widely accepted (Rauchensteiner et al., 2002; Ma et al., 2010). However, even

here some contradictory results can be found in the literature. In plant samples from Yugoslavia, Chalchat et al. (2000) could not identify chamazulene, but 1,8-cineole, chrysanthenon, and camphor are mentioned as main components of *A. collina*. Todorova et al. (2007) presented three chemotypes of this species (azulene-rich, azulene-poor, sesquiterpene-free types) based on the analysis of samples from six different populations in Bulgaria.

According to the literature references, the largest intraspecific variability could be devoted to *Achillea millefolium*. For this species, 19 different chemical compounds have already been mentioned in the essential oil as main components (Németh, 2005; Pecetti et al., 2012). Comparing the chamazulene content of the distilled oil, values between 0% and 85% have been detected by different authors (e.g., Figueiredo et al., 1992; Michler et al., 1992; Bélanger és Dextraze, 1993; Orav et al., 2001; Németh et al., 2007; Chou et al., 2013; etc.).

Taking into consideration only these data, we might assume that *A. millefolium* is an extremely variable species concerning its essential oil spectrum with numerous intraspecific chemical varieties. However, in this case, I would be more cautious because, in numerous references, the proper identification of the taxon is not obvious or botanical characterization is missing. The genus *Achillea* is a very complex one with species in a polyploid row, containing intrageneric sections and groups, many spontaneous hybrids, phenocopies, and aneuploid forms. Contradictory results may originate from a false definition of taxa belonging to the *A. millefolium* section only by morphological features or—on the other side—only by chromosome numbers. Similarly, investigation of non-representative samples like commercial samples, individuals of non-stable spontaneous hybrid or aneuploid character may lead to invalid information. Detailed morphological and cytological identification of any taxon belonging to the section *A. millefolium* seems to be a prerequisite for reliable chemical characterization; otherwise, comparison of the data is not really possible. In the last decade, molecular markers have also been developed for identification of certain taxa (e.g., Ma et al., 2010).

According to the above-mentioned information, unequivocal definition of the accessions showing diverse essential oil composition in the section *A. millefolium* as chemotypes could be more than questionable, and only references based on comprehensive determination of the investigated plant material can be accepted.

Nevertheless, the high variability concerning the composition of the essential oil of yarrow species is without doubt. Three chemotypes of *A. biebersteinii* were described from indigenous populations in Turkey based on the major compounds 1.8-cineole, p-cymene, camphor, piperitone and ascaridol (Toncer et al., 2010). According to Muselli et al. (2009) geographically distinct populations of *A. ligustica* also show chemically distinct characteristics. Corsican samples contain camphor (21%) and santolina alcohol (15%) as main compounds, Sardinian samples have *trans*-sabinyl acetate (18%) and *trans*-sabinol (15%), and those from Sicily can be characterized by high terpinen-4-ol (19%) and carvone (9%) accumulation. Similar results on other species are numerous.

A related species, wormwood (*Artemisia absinthium*), gained an adverse "reputation" due its thujone content and mutual side effects associated with absinthism (Lachenmeier et al., 2006). It is widely distributed in Europe and introduced also in other continents. The composition of the essential oil has been studied by several authors and highlighted that large amounts of thujones are representative only for one of the many chemotypes of *A. absinthium* while other mono- or sometimes sesquiterpenes are more frequently present as major components in the herb oil (Table 4.3). According to the investigation in the last decade, it is obvious that thujone may be not rarely even absent from the oil. Additionally, although wormwood is known among the few proazulen-containing species (Wichtl, 1997), chamazulene is only rarely and in low proportions present in the essential oil of the investigated accessions.

The real source of the polychemism in this species seems to be till now unknown. In our recent study based on the data of 12 different accessions, a connection between chemotype and habitat could not be justified in most cases. The majority of the accessions were heterogenous concerning appearance of chemotypes. The occurrence of thujone-type individuals was rather frequent in

TABLE 4.3
Main Components of Essential Oils from *Artemisia absinthium* Samples of Different Origin

Reference	Sample Origin	Determined Main Components/Chemotypes (Main Compounds in Area %)
Altunkaya et al. (2014)	Turkey	Myrcene (44%)
Arino et al. (1999)	Spain	*cis*-Chrysanthenyl acetate (31%–44%) + Cis-epoxyocimene (34%–42%)
Bagci et al. (2010)	Turkey	Chamazulene (29%)
Basta et al. (2007)	Greece	Caryophyllene-oxide (25%)
Chialva et al. (1983)	Italy	*cis*-Epoxyocimene (30%–54%) or β-thujone (41%)
	Romania	β-thujone (15%)
	France	Sabinyl acetate (32%) or chrysanthenyl acetate (42%)
	Siberia	Sabinyl acetate (85%)
Derwich et al. (2009)	Morocco	α-Thujone (40%)
Huong et al. (2018)	Spain	*cis*-Epoxyocimene (47%–76%)
	Belgium	α-Thujone (1%–52%) or β-thujone (25%–89%)
	Germany	β-Thujone (2%–85%) or *trans*-sabinyl acetate (1%–36%) or myrcene (4%–68%)
	Norway	*trans*-Sabinyl acetate (20%–78%)
	Hungary	Sabinene (2%–34%) + ß-myrcene (2%–42%)
	England	*cis*-Epoxyocimene (35%–65%) or isocitral (10%–49%) or sabinene (1%–38%)
Judzentiene and Budiene (2010)	Lithuania	*trans*-Sabinyl acetate (22%–51%) or α- and β-thujones (18%–72%)
Juteau et al. (2003)	Croatia	α-Thujone (49%) or *cis*-epoxyocimene (31%)
	France	*cis*-Chrysanthenyl acetate (34%) or *cis*-epoxyocimene (50%)
Llorens-Molina and Vacas (2015)	Spain	α-Fenchene (24%) or bornyl acetate (21%) or Myrcene (29%)
Lopes-Lutz et al. (2008)	Canada	α-Thujone (10%) + myrcene (10%) + Sabinyl acetate (26%)
Morteza-Semnani and Akbarzadeh (2005)	Iran	α-Thujone (70%)
Msaada et al. (2015)	Tunisia	Chamazulene (40%)
Mucciarelli et al. (1995)	Italy	*cis*-Epoxyocimene (25%) + *trans*-chrysanthenyl acetate (22%) + camphor (17%)
Nezhadali and Parsa (2010)	Iran	p-Cymene (10%) + camphor (15%)
Nin et al. (1995)	Italy	Terpinene-4-ol (29%)
	US	α-Thujone (70%)
Orav et al. (2006)	Greece	β-Thujone (38%)
	Estonia	β-Thujone (65%) or myrcene (30%) or Sabinyl acetate (71%)
	Russia	*cis*-Epoxyocimene (21%)
	Moldova	Myrcene (60%)
	Siberia	Sabinyl acetate (31%)
	France	Sabinyl acetate (85%)
	Armenia	Sabinyl acetate (34%)
Pino et al. (1997)	Cuba	Bornyl acetate (24%)
Rezaeinodehi and Khangholi (2008)	Iran	β-pinene (24%)
Sharopov et al. (2012)	Tajikistan	Myrcene (23%) + *cis*-chrysanthenyl acetate (18%)
Simonnet et al. (2012)	Switzerland	*cis*-Epoxyocimene (30%–40%)
Tucker et al. (1993)	US	α-Thujone (33%)

Note: +, indicates mixed chemotypes with more main components; or, indicates different chemotypes in the same accession.

TABLE 4.4
Chemotypes of *Tanacetum vulgare* According Selected References

Reference	Country	Chemotypes (Main Components)
Collin et al. (1993)	Canada	Camphor-cineole-borneol, β-thujone, chrysanthenone, dihydrocarvone
de Pooter et al. (1989)	Belgium	β-Thujone, chrysanthenyl acetate, camphor + thujone
Dragland et al. (2005)	Norway	Thujone, camphor, borneol, bornyl acetate, chrysanthenol, chrysanthenyl acetate, 1,8-cineole, α-terpineol
Forsen and Schantz (1971)	Finland	Chrysanthenyl acetate, isopinocamphone, not identified sesquiterpene
Hendrics et al. (1990)	Nether-lands	Artemisia ketone, chrysanthenol + chrysanthenyl acetate, lyratol + lyratyl acetate, β-thujone
Héthelyi et al. (1991)	Hungary	Yomogi alcohol, artemisia alcohol, davanone, lyratol + lyratyl acetate, chrysanthenol, carveol, carvone, dihydrocarvone, terpinene-4-ol, γ-campholenol, myrtenol, β-terpineol, 4-thujene-2-α-yl acetate, carvyl acetate, β-cubebene, juniper camphor, thymol, β-terpinyl acetate, linalool
Holopainen et al. (1987)	Finland	Sabinene, germacrene D
Mockute and Judzetiene (2004)	Lithuania	1,8-Cineole, artemisia ketone, camphor, α-thujone
Nano et al. (1979)	Italy	Chrysanthenyl acetate
Rohloff et al. (2004)	Norway	β-Thujone, camphor, artemisia ketone, umbellulone, chrysanthenyl acetate, chrysanthenone, chrysanthenol, 1,8-cineole
Sorsa et al. (1968)	Finland	α-pinene + tricyclene, β-pinene + sabinene, 1,8-cineole, γ-terpinene, artemisia ketone, thujone, camphor, umbellulone, borneol, humulenol

European samples, except a single one the accessions were not homogenous from this respect (Huong et al., 2018). No other works are suggesting any data on chemotype distribution except Chialva et al. (1983). Unfortunately, the plant material investigated by them included different plant parts, harvest years, samples distilled both fresh and dried, and originated either from natural habitats or from market. Under such conditions, the results cannot enable reliable conclusions, especially not in chemotaxonomic respect, although the title of the paper is suggesting this.

One of the earliest and most deeply studied plant species with respect to essential oil polymorphism has been tansy (*Tanacetum vulgare*). Formerly—due to the lack of reliable chemical-analytical investigations and systematic evaluation—it has been presented as a characteristic thujone containing species (Gildemeister and Hoffmann, 1961). Although it is true that this is the main component most frequently present in the essential oil, until today, the number of the detected main compounds in different chemotypes is near to 50. Some of these are summarized in Table 4.4. The dominant compounds are in most cases monoterpenes, but in some samples also sesquiterpene ones such as humulenole, germacrene D, or davanone were detected.

The spectrum of these monoterpenes is very wide. There are representatives of each types of the basic monoterpene skeletons except the carane group. Even if the main component itself is usually not enough for evaluation of the characteristics of the oil, tansy is a good example to illustrate the fact that the main compounds of different chemotypes may not necessarily belong to the same skeleton. It also means that they are not always products of closely related biosynthetic pathways, which might reflect a really heterogeneous genetic structure.

Large intraspecific chemical variability is by no means restricted to Asteraceae species. The genus *Thymus* comprises many species highly polymorphic for essential oil composition. Different chemotypes have been reported in at least 85 cases, mainly from the species *T. aestivus, T. herba-barona, T. hyemalis, T. mastichina, T. nitens, T. vulgaris*, and *T. zygis* (Stahl-Biskup and Sáez, 2003). For most of them, three to six intraspecific chemotypes have already been described.

Different chemotypes are often grouped as ones containing phenolic compounds and chemotypes with non-phenolic ones (Baser et al., 1993).

Common thyme, *Thymus glabrescens* Willd., is a procumbent dwarf shrub, indigenous on sunny hillsides of southeastern and central Europe. Recently, in Hungary, eight populations at different localities have been investigated and new chemotypes identified (Pluhár et al., 2008). Four chemotypes contained thymol as the main compound in the oil (15%–34%), but the second and third main compound has been different in each of them. One chemotype contained only monoterpenes as major constituents (*p*-cymene 45%, geraniol 14%, and linalyl acetate 10%) while two other ones only sesquiterpenes (germacrene D 55%, β-caryophyllene 15%, α-cubebene 51%). 1,8-cineole and thymyl acetate/carvacrol/*p*-cymene chemovarieties were described in Croatia; a terpinyl acetate chemotype was reported in Bosnia; and linalool/thymol/α-terpinyl acetate, geraniol, citronellol, and carvacrol chemovarieties were mentioned in Bulgaria (Pluhár et al., 2008). It can be established that in this species—in contrary to the formerly mentioned ones—the main compounds could be relatively well grouped based on their chemical constitution: acyclic monoterpenes, menthane skeleton group, and sesquiterpene ones. This led us to conclude that intraspecific differences in this species are primarily the results of diversity in biosynthesis at the level of terpene synthases and not in the following transformations.

Within a genus, different species may exhibit different levels of intraspecific chemical variability. The genus *Mentha* is a good example for this. Besides the best known species, *M. piperita*, there is only a small variability also in *M. pulegium.* While the first one is characterized always by the presence of menthol, the last one almost always contains pulegone as the main compound or one of the main compounds (Baser et al., 2012; Teixeira et al., 2012). The presence of piperitenon oxide in high percentages has been reported in each of the published studies for the oil composition of *M. suaveolens* (Baser et al., 1999, 2012; Božović et al., 2015). Similarly, *M. aquatica* seems to be a species of low essential oil variability. According to the available data, menthofuran has been detected in the huge majority of the investigated samples (Baser et al., 2012; Andro et al., 2013). On the other side, numerous species of the genus are really polymorphic concerning their volatile compounds. *M. longifolia, M. spicata*, *M. arvensis*, and also natural hybrids like *M. x dumetorum* exhibit a wide spectrum of essential oil compounds, and numerous chemotypes have been reported (Lawrence, 2007; Baser et al., 2012; Llorens-Molina et al., 2017).

4.2.2 Populations

During evaluation of the intraspecific essential oil variability of any species, one has to be aware of the fact that in many cases, the investigated plant material is far from a homogenous one. Although representative sampling is a prerequisite for these studies, unfortunately, this is only rarely the fact. It is still quite frequently not taken into account that different populations might reveal significant variability due to the individual differences of single plants. Description of differences among populations without referring to the individual variability within populations may lead to significant misinterpretation of data.

This is especially relevant for wild growing plants because natural populations are often heterogenous in many respects. A special difficulty is that the size of this diversity is not known either. Therefore, inadequate number of sampled individuals or bulked samples may obscure the real variability that can be demonstrated by several examples.

In natural stands of *A. crithmifolia*, considerable variability has been detected, and the level of several essential oil constituents varied on a large scale. Camphor (camphor above 50% in the oil), 1,8-cineole (this compound above 30% of the oil), and mixed-type individuals have been detected (Németh et al., 2000). It was found that the abundance of plant individuals belonging to the different chemotypes varied according to habitat. In this case, bulked samples could not tell us details about the real diversity of the stands, but individual sampling could reveal the three chemotypes present in these populations.

In a similar trial in Bulgaria, analyzing samples from seven habitats, besides camphorous- and 1,8-cineole-type individuals, an artemisia alcohol chemotype (with 24%–46% artemisia alcohol in the oil) has been described (Konakchiev and Vitkova, 2004). However, in this examination, the populations could not be characterized. and the abundance of the three chemotypes has not been described either, as only a single individual has been sampled from each habitat! Therefore, the results are only useful to provide data about the existing chemical diversity of the species but not about their frequency and distribution.

Unfortunately, some other references are even more questionable if they could give appropriate information on natural variability of this species. Bulked plant material from a Serbian population "near Niš" was characterized by high (19%) proportions of *trans*-chrysanthenyl acetate (Palić et al., 2003), while another in Greece "from Pilio mountain at the altitude of 700 m" by larger levels of α-terpineol (Tzakou et al., 1993). These data do not tell us anything about the quality of the oil of single individuals where these ratios might be much lower or higher, respectively. A single sample from a population might lead to false interpretation not only from theoretical point of view but also about practical/pharmaceutical value of these stands because the representativeness is at least questionable.

In the same genus, significant amounts of chamazulene are generally present in the essential oil of *A. collina*. Rarely can we find, however, any reference about the individual distribution of this compound inside a plant population, although collection of bulk samples may again lead to false consequences and cannot represent a basis for standard drug quality.

Table 4.5 shows that among 23 Hungarian *A. collina* populations, differences of mean values varied from 33.2% till 67.1% while the standard deviations show twelvefold differences! A population with 1.8% standard deviation ("Diósd") means, in the practice, a strongly homogenous stand where the high level of chamazulene manifests itself in almost each individual. On the other side, a population like "Alsótold," of similar mean value but with a much higher standard deviation, can be evaluated as an unstable one, less suiting even for commercial purposes. A more detailed investigation afterward revealed that the mentioned results could be traced back to individual differences. The plants in the examined wild populations of *A. collina* could be sorted in four groups based on the characteristic spectrum of the essential oil. Individuals, accumulating chamazulene in high proportions as the absolutely main component of the oil are clearly different from the ones having both β-caryophyllene and chamazulene in higher levels. Individuals of only low levels of chamazulene and having other compounds as major ones form a distinct group while the plants with essential oil lacking chamazulene are sorted in the fourth group. The evaluated mean values of the populations obviously reflect the proportion of these chemotypes (Németh et al., 2007).

As discussed above, more than twenty chemotypes of wormwood (*Artemisia absinthium*) have been described until recently in the literature. Checking the methods of the published papers, it can, however, be established that in the huge majority of the cited references, the method of sampling has only be described as follows: "aerial parts/leaves/plants were collected …" without providing any information about the number of individuals, replications, or the amount of the sample. On the other side, a paper mentioned "four different plants" which have been harvested, but in this case the low number of individual plants is surely not able to represent either the population or its variability. Intrapopulation variability has been studied only in exceptional cases. Llorens-Molina et al. (2016) presented the common occurrence of two well-distinguishable chemotypes (*cis*-beta-epoxyocimene above 70% of essential oil and *cis*-beta-epoxyocimene at 60%–70%, and with *cis*-chrysanthenyl acetate at 10%–20%) in a wild habitat in Spain. The two chemically—and presumably also genetically—distinct individuals are distinguishable only by EO analysis and do not show any external marker traits. The authors called the attention to the importance of individual monitoring during examination oil composition because of the obvious differences among plants of the same population. Similarly, the detailed study Huong et al. (2018) on 120 individual samples

TABLE 4.5
Average Values and Standard Deviations of the Essential Oil Content and its Chamazulene Level in 23 Spontaneous Hungarian *Achillea collina* Populations

	Essential Oil Content Of Flowers (% d.w.)		Chamazulene Content in Flower Oil (ess. Oil %)	
Population (origin)	**Mean**	**Std. Dv.**	**Mean**	**Std. Dv.**
Alsótold	0.55	0.49	53.6	25.3
Apc	0.27	0.06	45.7	15.4
Aszód	0.48	0.22	63.1	12.5
Balatonakali	0.36	0.11	67.1	4.2
Balatonudvari	0.29	0.07	61.0	7.2
Bokor	0.35	0.13	64.5	5.8
Csepreg	0.20	0.08	40.0	18.3
Csillebérc	0.33	0.22	60.3	7.2
Diósd	0.42	0.11	61.0	1.8
Jobbágyi	0.33	0.18	33.7	24.6
Kevélynyereg	0.30	0.13	60.7	7.6
Lupasziget	0.31	0.09	52.5	4.8
Makkoshetye	0.18	0.08	40.3	28.1
Mezőnyárád	0.27	0.14	33.2	18.9
Mikóújfalu	0.44	0.07	57.7	8.9
Nagymaros	0.71	0.36	47.8	22.9
Nagymaros	0.53	0.15	64.2	5.0
Oroszlány	0.33	0.11	60.7	2.8
Solymár	0.37	0.08	58.3	5.1
Sopron	0.37	0.29	31.3	26.9
Szigliget	0.35	0.19	47.5	19.2
Tiszavasvári	0.65	0.58	30.5	30.4
Zenta	0.47	0.12	44.7	22.4
Mean	0.39	0.24	51.3	19.3
SD value	0.334	–	25.4	–
P level	0.005	–	0.000	–

Source: Modified from Németh, É. et al. 2007. *J. Herbs, Spices Med. Plants*, 13: 57–69.

of 12 accessions provided a well-established base for chemotype definition and characterization of populations (Table 4.6). In this work, it was also demonstrated that differences among individuals manifest themselves not only in the main components but also in the total spectrum. The varying ratios of mono- and sesquiterpene compounds to each other demonstrate it very well: individuals with 89% monoterpenes and 11% sesquiterpenes represent one marginal value while, on the other side, an individual with 10% monoterpenes and 90% sesquiterpenes in the essential oil express in GC peak area percentages is the contrast.

Sampling of a population of *Thymus longicaulis* subsp. *longicaulis* in Turkey resulted in distinguishing three different chemotypes: thymol type, geraniol type, and α-terpinyl acetate types. It was shown that individuals belonging to the different chemotypes can be found near to each other even on a one-square-meter area (Baser et al., 1993).

The above examples represent quite well that in a chemically diverse species in consequence of the large plant-to-plant variability, the populations may be heterogeneous too.

TABLE 4.6
Distribution of the Identified Chemotypes in Twelve Wormwood (*Artemisia absinthium*) Accessions

Chemotype	Proportion in the Accessions (%)											
	Bel	**Eng**	**Ger0**	**Ger1**	**Ger2**	**Hum**	**HuW1**	**HuW2**	**HuW3**	**HuW4**	**Nor**	**Spa**
Pure Chemotypes (Main Component >30% of Total Gc Area)												
Thujone	**100**			30						40	20	
cis-Epoxyocimene		20					10	30				**100**
trans-Sabinyl acetate			10	10	**100**				10		40	
Sabinene		10										
ß-Myrcene				20		20	10	10	30			
Linalool										10		
cis-Chrysanthenol						10						
(*Z*)-*Iso*-citral		10		10								
Selin-11-en-4-α-ol								10				
(*E*)-Nuciferol isobutyrate			10									
Mixed Chemotypes (Two or Three Components >30% of Total Oil)												
Thujone + *cis*-epoxyocimene											10	
Thujone + *cis*-epoxyocimene + *trans*-sabinyl acetate											20	
Thujone + *trans*-sabinyl acetate											10	
Sabinene + ß-Myrcene		20	30				40		10			
ß-Myrcene + β-caryophyllene				10		10	10	20				
ß-Myrcene + (*Z*)-nuciferol isobutyrate			10									
Linalool + β-caryophyllene				10				10				
Linalool + (*Z*)-nuciferol isobutyrate										30		
β-Caryophyllene + selin-11-en-4-α-ol						20		20				
Selin-11-en-4-α-ol + (*Z*)-*iso*-citral												
Selin-11-en-4-α-ol + (*Z*)-nuciferol isobutyrate						10			10			
Other composition	0	40	40	10	0	30	30	0	40	20	0	0

Source: Modified from Huong et al. 2018. *J. Essent. Oil Res.*, 30: 421–430.

4.3 CONNECTIONS OF CHEMICAL DIVERSITY WITH OTHER PLANT CHARACTERISTICS

4.3.1 Propagation and Genetics

The homogeneity or variability of a population often stays in connection with the usual propagation method of the species. Phenotypic manifestation of diverse genetic background and appearance of different chemotypes in a plant stand can be supported by sexual propagation and cross pollination. To the contrary, vegetative propagation or autogamy enhances uniformity of the population.

Vetter and Franz (1998) proved the large degree of self-incompatibility in five *Achillea* species (*A. ceretanica, A. collina, A. pratensis, A. distans*, and *A. monticola*). While the number of seeds in cross-pollinated flowers reached 47–110 pcs, it was solely 0–11 pcs in self-pollinated ones. Our long-term practical experiences with yarrow ascertain this finding and it is in obvious coincidence with the large intraspecific chemical diversity of these species.

Xenogamy is the preferred way of fertilization in several important medicinal species. As an example, *Lamiaceae* species are cross-pollinating ones based on the morphological constitution of the flowers and the mechanism of proterandry. Beside xenogamy, geitonogamy may occur between flowers of the same plant; however, seed-set rates are much lower in this case (Putievsky et al., 1999; Németh and Székely, 2000). In some species of the same genus, both hermaphrodite and male-sterile flowers can be found. In thyme (*Thymus vulgaris*), it has been described that the latter ones occur primarily in suboptimal environments, assuring that outcrossing enhances fitness of the progenies while the hermaphrodite flower structure enables autogamy. Depending on the type of fertilization, the essential oil pattern varies characteristically (Gouyon et al., 1986).

Species that are generally propagated by vegetative methods like peppermint, tarragon, etc. do not show any or only a minimum variability among individuals. This fact sometimes is considered as an adverse phenomenon and an obstacle in effective selection and genotype improvement. Therefore, breeders usually try to increase the variability of these plants with specific methods. Mutation breeding proved to be a prosperous tool in producing wilt-resistant strains of peppermint in the US (Murray et al., 1986). Induction of polyploids by colchicine and the crossing of fertile accessions afterward has been the basics in developing the highly productive variety "Multimentha" in East-Germany (Dubiel et al., 1988). Development of new chemical varieties is endeavored today more and more by molecular genetic methods (Croteau et al., 2005; Wagner et al., 2005).

On the other side, clonal propagation is an optimal way to produce chemically homogenous populations for commercial production and processing purposes. According to my own experiences, seed sowing of tansy results in an enormous segregation of the population which is not acceptable as raw material for industrial utilization. Therefore, vegetative propagation by young shoots has been elaborated for the production of selected chemotypes (Zámboriné et al., 1987).

The chemical heterogeneity of several wild-growing populations seems to be today one of the basic motivations for introduction of economically important wild species into the agriculture and selection of their stable varieties. Breeding is going on usually parallel with development of technological methods.

Fennel has been cultivated already for many decades and selected cultivars are registered in numerous countries. The main goals of the breeding have been definitely the increase of essential oil content and stabilization of its composition. During maintenance of our cultivar "Foenipharm," we checked the most important characteristics of individual mother plants. The results show that deviations among the plants are minimal owing to the long-term breeding and variety maintenance process (Table 4.7).

Breeding of the polymorph species *Artemisia absinthium* in the Conthey Research Centre (Switzerland) resulted in a uniform variety accumulating *cis*-epoxyocymene as the main compound. After screening of more than 800 plants from 24 accessions originating from six countries, the researcher selected and stabilized the desired chemovariety (Simonnet et al., 2012).