Enantioselective Gas Chromatography in Flavor and Fragrance Analysis: Strategies for the Identification of Known and Unknown Plant Volatiles

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Abstract

This review describes current analytical technology for the analysis of chiral constituents in essential oils and other natural volatiles, flavor and fragrance compounds, and covers some important results achieved by natural compound chemists, food chemists, perfumers, and molecular biologists. The technique of enantioselective gas chromatography (GC) is described and applied for assigning absolute configuration of chiral natural compounds, which is strongly connected to differences in odor properties of their enantiomers. In addition, some recent results to facilitate the handling of GC–mass spectrometry data of known and unknown plant volatiles are discussed.

Introduction

Natural volatiles occur in a tremendous number and variety as constituents of higher and lower plants, microorganisms, fungi, insects, and marine organisms. Terpenes, aliphatic and aromatic compounds resulting as secondary metabolites from different biogenetic pathways, are among the most common constituents (1). They may be present as different stereoisomers (enantiomers or diastereoisomers), which increases the number of possible structures and complicates their correct identification. Volatiles play a key role in plant–insect communication and may be active in minute amounts in a specific organism. Their biological activity may greatly depend on stereochemical properties. Moreover, the unambiguous identification of minor constituents in a complex mixture is a great challenge for the analyst. This means that high sensitivity and efficiency, together with high selectivity, are major demands for an analytical system. As far as volatile compounds are concerned, high efficiency separation of constituents, sensitivity, and selectivity can usually be achieved by capillary gas chromatography (GC) coupled to mass spectrometry (MS). Because MS characteristics are not always sufficiently unique, GC retention indices should be used as a second dimension for data comparison. Mass spectral and retention data can be documented in data libraries and computer software is available for (semi)automatic data comparison. In this way a complex mixture of volatiles (essential oils or natural extracts) can be systematically and rapidly screened for compounds that are already known. For the investigation of stereochemical details (i.e., absolute configuration) a third analytical dimension can be introduced by applying enantioselective GC with chiral stationary phases mainly based on cyclodextrin derivatives (1–8). For this technique, however, both enantiomers of a chiral compound should be available for comparison and assignment of the elution order of a pair of enantiomers. In addition, multidimensional GC techniques employing a combination of a conventional capillary column and an enantioselective column (9,10) and the recently established so called "comprehensive" GC methodology (11) may support resolution and unambiguous identification of constituents in a multicomponent mixture.

Particularly demanding is the identification of unknown (new) compounds. In most cases, the isolation of a single constituent from a complex mixture and investigation by one- and two-dimensional NMR techniques is inevitable. For the isolation, micropreparative chromatographic techniques such as GC, high-performance liquid chromatography, and thin-layer chromatography (plates precoated with Ag^+ ions may be used for separation of double bond isomers) can be successfully applied.

Because the accessibility of samples from natural sources may be limited (a typical example is the identification of sesquiterpenes from biosynthetic studies), the process of structure identification must be optimized to microscale procedures to get the best results. A strategy for the identification of known and unknown natural volatiles will be presented in this review.

Discussion

Essential oils

Preparation and collection methods

Volatile plant constituents are obtained as essential oils by various methods of hydrodistillation (or steam distillation) of leaves,

flowers, stems, roots, bark of aromatic plants, or by cold expression of the peel in the case of citrus fruits (lemon, orange, bergamot, etc.). As an alternative, solvent extraction methods at room temperature and normal pressure or enhanced temperature and pressure conditions (accelerated solvent extraction) can be used. Supercritical fluid extraction is applied as a particularly mild procedure for the preparation of extracts of spices and precious fragrances. In recent years solid phase microextraction (SPME) has become a valuable tool for the analysis of volatiles from aqueous solutions or directly from the space above (headspace analysis, solvent-free extraction) (12,13). Lipophilic volatiles are preferentially adsorbed on a polymer (polysiloxanes of different polarities) coated fused-silica fiber. The adsorbed material is then directly transferred and desorbed into the hot injection port of the GC. This is a very direct, rapid and mild, but sometimes largely discriminating, analytical procedure. A very instructive report on SPME in essential oil analysis was given by Kubeczka (14). It was demonstrated that the essential oil even from single oil compartments (secretory glands) of a plant could be collected and transferred to the GC. Another interesting approach described by Kubeczka is the collection of a single constituent at the end of the GC column (detector site) with the SPME device and its transfer to a second GC equipped with an enantioselective column for enantiomeric analysis.

For efficient collection and subsequent analysis of fragrances from living plants and flowers (15,16) or the very small amounts of volatiles generated during biosynthetic studies in certain time periods, for example, as response of a plant to herbivorous damage (17), a "closed-loop" adsorption technique, offering an extraordinary accumulation factor, has become very popular (18).

Different isolation procedures naturally result in differences in the relative composition of the products because the extraction power of the applied solvents and the applied pressure and temperature parameters have a strong influence on the yield of single constituents of the essential oil. Supercritical CO_2 is comparable to *n*-hexane in its polarity and, therefore, may preferentially extract unpolar constituents, yet hydrodistillation may be more representative of the total mixture but may cause partial decomposition and rearrangement processes in the case of labile plant constituents. On the other hand, organic solvents will extract not only volatiles but also plant waxes, fatty oils, and high boiling lipids that tend to contaminate the GC column. The advantages and disadvantages of hydrodistillation, which probably is still the most reproducible method, have been discussed many times. Certain facts were documented in the literature. Thus, only the (*R*)-(–)-enantiomer of linalool (**1**) is present in lavender extracts (*Lavandula angustifolia*), but partial racemization of this fragrant constituent will occur during the slightly acidic conditions during prolonged hydrodistillation (19). Moreover, the occurrence of enantiomeric mixtures of α-terpineol (**2**) may be ascribed to rearrangement of linalool. α-Terpineol may also be formed during hydrodistillation under acidic pH values by hydration and rearrangement of a-pinene (**3**), but terpinen-4-ol (**4**) is formed from sabinene (20,21). Cope rearrangement, a skeletal rearrangement, is known to take place in the case of labile sesquiterpenes with a 1,5-disposition of double bonds [germacrene A (**5**), B (**6**), C (**7**), and their derivatives] (22). Germacrene D (**8**), a sesquiterpene hydrocarbon and very common constituent in many plants, has a high tendency to rearrange to a large variety of products under acidic conditions or under heat or irradiation by light (23). Moreover, rearrangement processes may also take place in the hot parts of the GC (injection port or column) or may be caused by active surfaces in the GC system. Nevertheless, the products obtained by a standard hydrodistillation procedure are considered as "natural essential oils" as long as accepted, standardized procedures are used for their preparation.

Proof of authenticity and adulteration

As essential oils have a high commercial relevance for the flavor and fragrance industry and are used in many fields including perfumery, cosmetics, food technology, pharmaceuticals, phytomedicine, aromatherapy, etc.; the optimization of profits by using cheaper artificial (synthetic) material added to the natural essential oil is unfortunately a logical consequence. In other cases precious essential oil products may be adulterated by the addition of cheap volatiles from other natural sources to increase yield and profit. The most frequent cases of "systematic" adulterations are found for lavender oil (*Lavandula angustifolia*, syn. L. *officinalis*) (19,24) bergamot oil (*Citrus bergamia*) (25,26,31) (Figure 1),

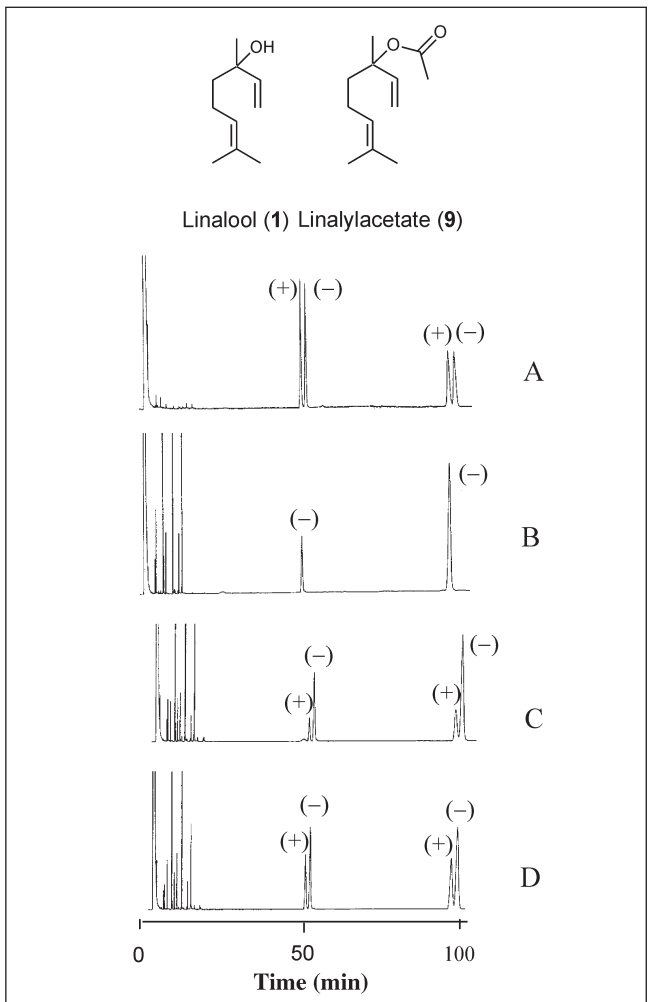

Figure 1. Enantiomer separation of linalool (**1**) and linalyl acetate (**9**): (A) racemate, (B) natural bergamot oil, and (C and D) adulterated bergamot oil. For the figure, 25 m of octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin (50% in OV 1701, w/w) at 55°C.

balm oil (*Melissa officinalis)* (27–29), rose oil (from *Rosa damascena* in Turkey, *R. centifolia* in Morocco) (30,31) (Figure 2) or peppermint oil (*Mentha piperita*) (25–29). In all of these cases, enantioselective GC may be the method of choice to prove these manipulations (25). Lavender oil may be adulterated by mixing with cheaper essential oils of other *Lavandula* species (lavandin oil, spike oil) with different compositions of their constituents or by the addition of racemic linalool and linalyl acetate (19,24). Such adulterations can be accompanied by traces of dihydro- and dehydrolinalool from the technical process. Bergamot oil is an expensive product (~ 120 euro/kg) produced at a relatively low crop/year and the demand by the fragrance and flavor industry was compensated by adding some percentage of the "unnatural" (racemic) linalool (**1**) and linalyl acetate (**9**). Only the enantiomerically pure (*R*)-enantiomers of both compounds are major constituents of natural bergamot oil (Figure 1). Several cyclodextrin derivatives can be used for enantiomer separation of linalool and linalyl acetate for proving this adulteration (31).

A particularly expensive essential oil is produced from the fragrant herb *Melissa officinalis* (balm oil). The relatively low yield obtained by hydrodistillation and low production rate of 50–80 kg/year in Europe may be responsible for the high price (~ 5,000 euro/kg). The genuineness of balm oil can be proven by the enantiomeric purity of the constituent (–)-citronellal, which should be close to 100% (20). Most commercial samples (12 out of 16) were found to contain minor or larger amounts of (+)-citronellal (**10**), probably added as a constituent of cheap citronella oil (*Cymbopogon winterianus*), which contains enantiomeric mixtures of citronellal (**10**) with an excess of the (+)-enantiomer. Again, enantioselective GC can help to assess the quality of the expensive product (20–23,25) (Figure 2).

Peppermint oil can be considered as a mass product (~ 5,000 tons/year) for the pharmaceutical, flavoring, food, and cosmetic industries. However, there are different qualities of this essential oil on the market with quite a bias in price. Real peppermint (*Mentha piperita*) oil has a more flowery, pleasant flavor than so called "mint oil" (*Mentha arvensis*) or "cornmint oil" (after partial dementholization), the latter being approximately 4 times less expensive than peppermint oil. Both qualities can be distinguished by certain GC criteria (32–35). The most important seems to be the occurrence of (–)-isopulegol (**12**) at a level greater than 0.07% (which is the average for real peppermint oil) up to 1.2–2.0%, which is indicative of *Mentha arvensis* (33) (Figure 3). But another constituent, (+)-*trans*-sabinene hydrate (**15**), which is present in real peppermint oil (~ 1%), is close to zero in *M. arvensis*. Other less common adulterations of *Mentha piperita* oil can be detected by investigating the enantiomeric purity of (–)-menthyl acetate (**13**), a constituent that is present in *M. arvensis* in very small amounts (~ 1%), but *M. piperita* oil should contain 2–8% of this constituent. In this case, the presence of the (+)-enantiomer indicates the addition of synthetic material (34) (Figure 4).

The presence of synthetic flavor compounds can independently be proven by the determination of the C^{13}/C^{12} ratio for GC

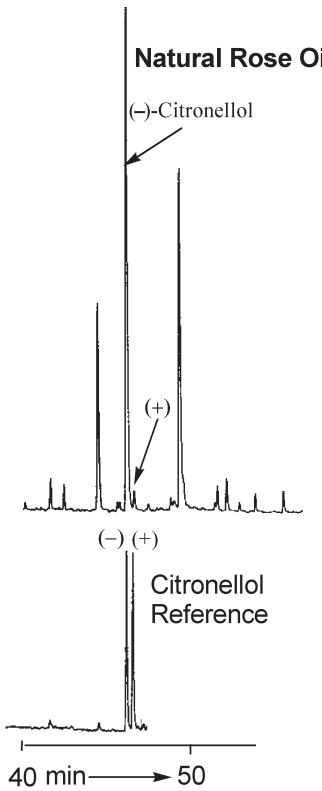

Figure 2. Enantiomer separation of citronellol (**11**) and determination of the enantiomeric purity of (–)-citronellol in rose oil. For the figure, 25 m of heptakis(2,3-di-*O*-acetyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (50% in PS 086, w/w); 65°C, 5 min isothermal, then 1°C/ min.

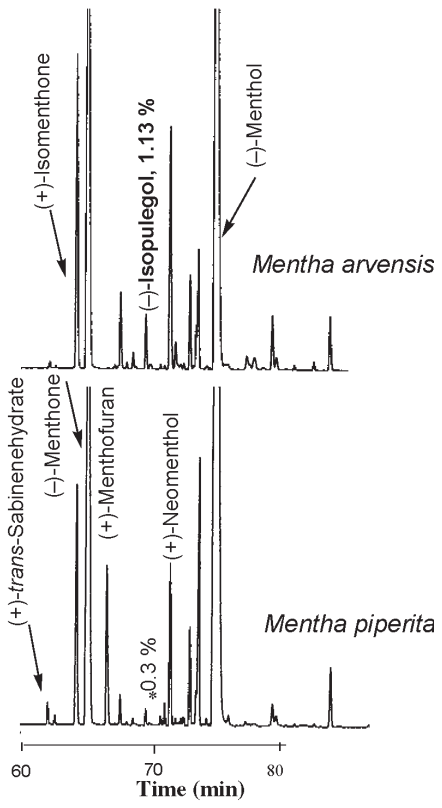

Figure 3. Determination of (–)-isopulegol (**12**) in *Mentha piperita* and *M. arvensis* essential oils. A typical value for *M. arvensis* is 1.13% (–)-isopulegol. For the figure, 25 m of octakis(6-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin (60% in OV 1701, w/w); 30°C for 20 min, then 1°C/min to 160°C.

resolved peaks by isotope ratio MS (IRMS) (28,35–37).

The majority of the most important fragrance and perfume compositions is based on natural rose oil (38), with its extraordinary, precious fragrance consisting of at least 300 constituents (39). The low yield and expensive production procedures are the reason for the very high price of genuine rose oil. Again, enantioselective GC can differentiate genuine rose oil from cheaper substitutes. Some of the most important constituents of rose oil are (–)-citronellol (**11**), (–)-(2*S*,4*R*)-*cis*- (**16**), and (–)-(2*R*,4*R*)-*trans*-rose oxides (**17**). These three constituents are biogenetically related to each other and enantiomerically pure (31,40). Only a few cyclodextrin derivatives, for example 2,3-di-*O*-acetyl-6-*O*-t.butyldimethylsilyl-β-cyclodextrin (40), are suited for resolving the enantiomers of these constituents in only one GC analysis. A recently described monofunctionalized cyclodextrin derivative 3'-*O*-acetyl-2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl-β-cyclodextrin proved particularly useful for this task (41).

Chamomile oil (*Chamomilla recutita*, syn. *Matricaria chamomilla*) is also a high-priced essential oil with great therapeutic relevance because of its anti-inflammatory properties. The major constituent, which is believed to be the main active principle, is (–)-α-bisabolol (**18**) with high enantiomeric purity (42). Because of its high price (~ 1,000 euro/kg) and the availability of cheaper substitutes chamomile oil has been adulterated by adding essential oils from other plants that may also contain

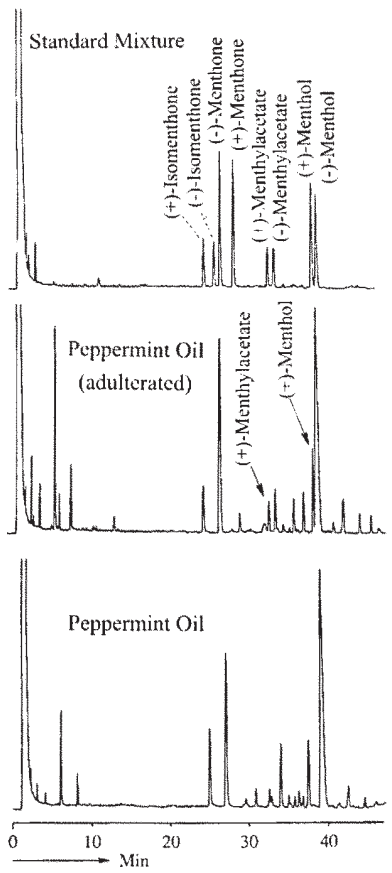

Figure 4. Adulteration of peppermint oil by addition of racemic menthyl acetate (13) and menthol (14). For the figure, 25 m heptakis(2,3-di-*O*-acetyl-6-O-t.butyldimethylsilyl)-β-cyclodextrin (50% in polysiloxane 86, w/w); 65°C, 5 min isothermal, then 1°C/min to 110°C.

α-bisabolol but as different stereoisomers (43). α-Bisabolol occurs in four stereoisomeric forms (44), however, only one stereoisomer is present in chamomile oil (Figure 5).

In a recent investigation of some common essential oils such as caraway oil (*Carum carvi*), lavender oil, neroli oil (hydrodistillation product of freshly picked blossoms of *Citrus aurantium*), and coriander oil (*Coriandrum sativum*), adulterations were detected (45). In natural caraway oil, less than 1% of (–)-carvone but > 99% of the optical antipode is present. An established quality control procedure is documented (46). The detection limit for (–)-carvone in caraway oil is less than 1% if an appropriate cyclodextrin column [e.g., octakis(2,6-di-*O*-methyl-3-*O*-pentyl)-γ-cyclodextrin or hexakis(2,3,6-tri-pentyl)-α-cyclodextrin] is used (5). Neroli oil is expensive (> 1,000 euro/kg) and is frequently adulterated by the cheaper petitgrain oil prepared by hydrodistillation of the leaves and twigs of the same plant, but adulterations based on the enantiomeric proportions of linalool in hydrodistillation products are sometimes hard to prove. This is also true for coriander oil with a (natural) large excess of the (*S*)-(+)-enantiomer of linalool (Figures 5 and 6).

Stereochemistry and flavor

Chirality plays an eminent role in odor and flavor perception. The odor intensity and sensorial impact between enantiomers may vary from extremely low perception levels of one enantiomer to odorless of the other enantiomer. More commonly, both enantiomers may more or less differ in their odor qualities (47). Reviews on these aspects of flavor analysis were reported recently (47, 48–50). Therefore, enantioselective GC in combination with a "sniffing port" can be used for organoleptic odour differentia-

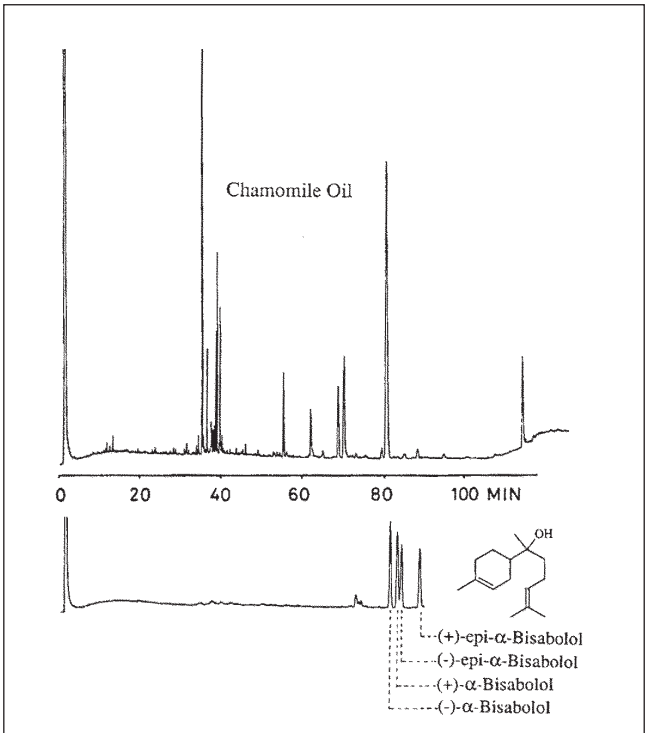

Figure 5. Separation of the four stereoisomers of α-bisabolol and proof of enantiomeric purity of (–)-α-bisabolol (**18**) in chamomile essential oil. For the figure, 25 m octakis(2,6-di-*O*-methyl-3-*O*-pentyl)-γ-cyclodextrin (50% in OV 1701, w/w); 50°C, 2°C/min to 130°C.

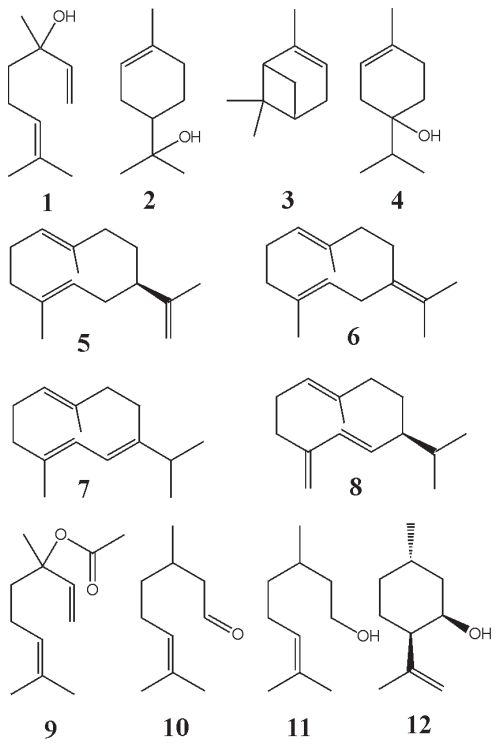

Figure 6. Compounds 1–12.

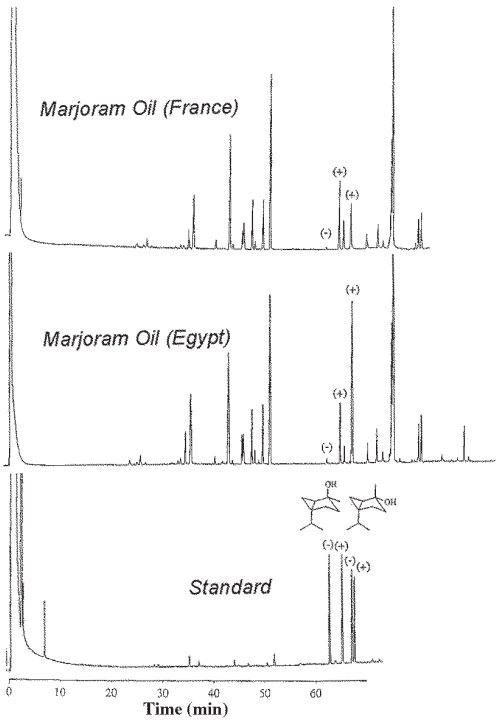

Figure 7. Enantiomer separation of four stereoisomers of *cis*- and *trans*-sabinene hydrate (**15**) and enantiomeric composition of these compounds in two chemotypes of marjoram oils. For the figure, 25 m octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin (60% in OV 1701, w/w); 30°C for 20 min, then 1°C/min to 160°C.

tion of stereoisomers not only of natural but also of synthetic origin (48–54).

A large majority of essential oil constituents belongs to the group of terpenoids. Many of them are chiral compounds, and either one of the two enantiomers or enantiomeric mixtures or, in case of more than one stereogenic center, diastereomeric mixtures of both may be present in natural plant volatiles. In fact, the product specificity of terpene synthase enzymes seems to increase from monoterpenes (55,56) (Figures 7 and 8)—most of them are present in essential oils as enantiomeric mixtures and enantiomeric proportions may serve as a "fingerprint" for a specific essential oil provenience—to sesquiterpenes (57), to diterpenes (58).

Only few sesquiterpenes were found to occur as mixtures of enantiomers. Their proportions can be directly determined even from very complex mixtures (Figure 9) by two-dimensional GC by transferring small sections of a GC peak from a conventional capillary column to an enantioselective capillary column. Examples are given in the literature (59–61). The enantiomers of sesquiterpene hydrocarbons can be very well resolved on capillary columns with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β- and γ-cyclodextrins or heptakis(2,3-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (60).

A very unusual case is the presence of both germacrene D enantiomers (8) in the higher plant *Solidago canadensis* (62). This observation was very important as germacrene D can be easily rearranged to many other sesquiterpene hydrocarbons (23). Starting from a certain enantiomeric proportion of germacrene D it was possible to generate a series of reference compounds with

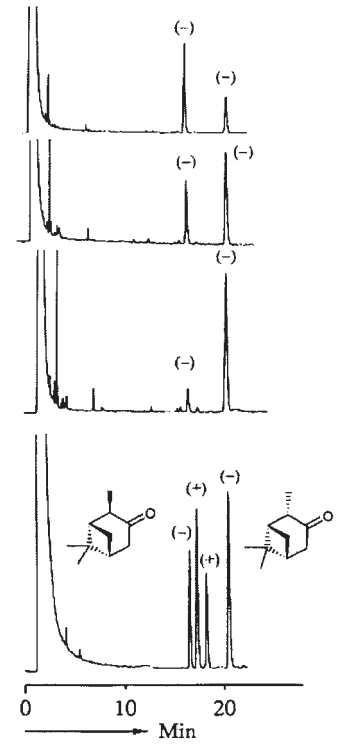

Figure 8. Enantiomer separation of isopinocamphone (**19**) and pinocamphone (**20**) in several chemotypes of hyssop oil. For the figure, 25 m heptakis(3'*O*-acetyl-2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (50% in OV 1701; 65°C) (41).

the same (or very similar) enantiomeric composition because these rearrangements usually proceed strictly stereospecifically. Access to both enantiomers of a sesquiterpene, which is necessary for the determination of the elution order on the enantioselective column and for assigning the absolute configuration, is otherwise a serious problem. It can only be solved by synthesis of specific isomers (enantiomers) or by isolation of the "unusual" enantiomer from a lower organism [liverworts (*Hepaticae*), marine organisms, fungi, etc. (Figures 9 and 10)]. After enantiomeric mixtures of many sesquiterpenes could be prepared quite easily, preparative scale GC with modified cyclodextrins was established for isolation of single enantiomers in the miligram range (63). In the case of diterpenes, so far, only one case was reported in which both enantiomers of sclarene are present in the higher plant *Araucaria heterophylla* (64) (Figures 10–12).

Many chiral plant volatiles and a great variety of aroma compounds of all kinds of substance classes have been analyzed using modified cyclodextrins as chiral stationary phases since their introduction in 1987. Reviews have been published regularly in this field (65–69). The fragrance and flavor industry has stimulated the development of analytical methods of chiral analysis after the correlation of absolute configuration and odor quality and intensity was recognized. This resulted in great intensifying of efforts toward asymmetric synthesis of enantiomerically pure fragrance and flavor compounds. Some of this work was nicely reviewed by Ohloff (70) and Frater et al. (71). A treatise of synthetic fragrance chemistry is also found in the monograph of Teisseire (72).

A series of important fragrance compounds belong to the group of "irregular" terpenoids as their biosynthesis does not follow the "biogenetic isoprene rule" (73). Among those compounds with exceptional fragrance characters are the ionones [in raspberries, black tea (*R*)-α-ionone (**25**), flower scents (e.g., violets)], irones, fragrant principle of natural *Orris* rhizomes oil from *Iris germanica* [(–)-*cis*-α-irone (**26**), (–)-*cis*-γ-irone (**27**)] or *I. pallida* [(+)-*cis*-α-irone, (+)-*cis*-γ-irone)] (Figure 13), and damascones [α-damascone (**28**)] in black tea (*Camellia sinensis*). Other derivatives of this group of megastigmanes (C-13-isoprenoids) are found in the flowers of *Boronia megastigma* [3-hydroxy-megastigm-7-en-9-one (**29**)] and *Osmanthus fragrans* and as theaspiranes (**30**) (in black tea, raspberry, and many fruits), theaspirones (**31**) (from quince juice, *Cydonia oblonga*), vitispiranes (**32**) (in vine), or edulane derivatives (passion flower, *Passiflora edulis*). Some of them are highly appreciated in the perfumery industry.

Most of these compounds have been resolved by enantioselective GC using cyclodextrin derivatives. Thus, the absolute configuration of α-ionone (**25**) and α-damascone (**28**) from black tea infusions was determined as early as 1989 (74). The different odor and taste impressions of single enantiomers were also documented (48,75). Irones and dihydroirones are present in the essential oils of *Iris* rhizomes as mixtures of enantiomers and double bond isomers in very different proportions. This greatly influences its commercial value and applicability in perfumery. Again enantiomeric resolution (Figure 13) and sensorial evaluation resulted in tremendous differences in odor descriptions

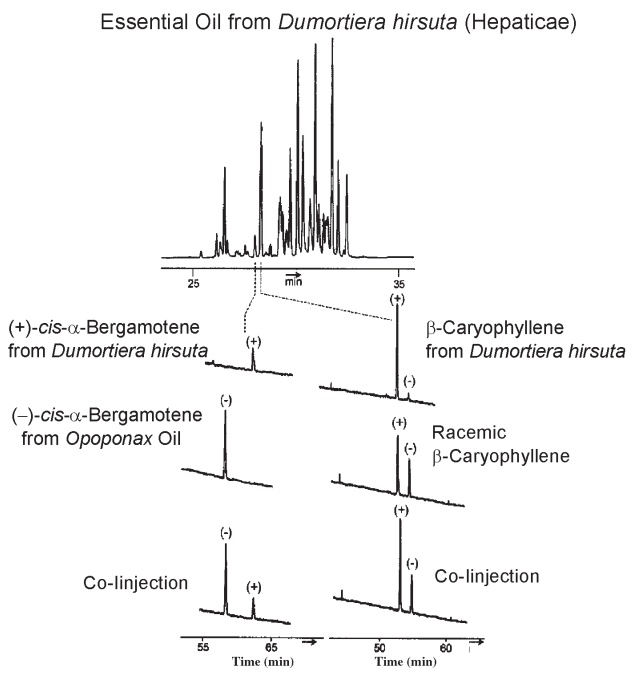

Figure 9. Two-dimensional enantioselective GC investigation of two peaks of the sesquiterpene hydrocarbon fraction (top; 25-m capillary with PDMS CPSil-5, 60°C, 3°C/min to 200°C) of the essential oil of the liverwort *Dumortiera hirsuta* and peak transfer to a 25-m capillary with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin at 100°C. As expected, the unusual enantiomers (+)-*cis*-α-bergamotene (**21**) and (+)-*trans*-β-caryophyllene (**22**) are present.

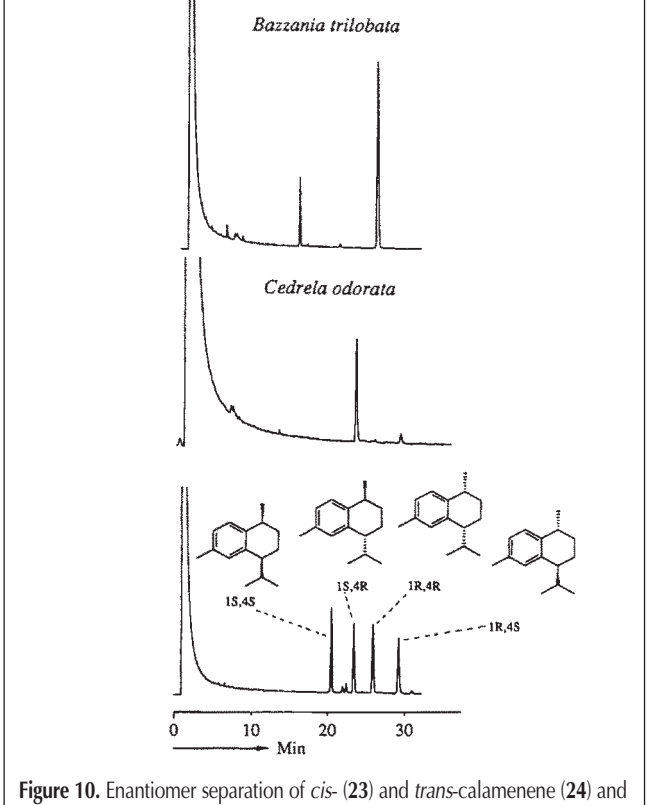

Figure 10. Enantiomer separation of *cis*- (**23**) and *trans*-calamenene (**24**) and assignment of absolute configuration in essential oils of the liverwort *Bazzania trilobata* and the higher plant *Cedrela odorata*. For the figure, 25 m heptakis(2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl-β-cyclodextrin at 120°C.

(48,76,77). The theaspirane enantiomers were first resolved by Guichard et al. (78). After preparative GC resolution, using a thick-film capillary column with permethyl-β-cyclodextrin, the different isomers of theaspiranes (79) and theaspirones and vitispiranes (80) from several natural sources were evaluated with respect to the relationship of absolute configuration and sensory impression (68,79).

Flower scents

In his fascinating monograph on the scent of orchids, Kaiser (15) has contributed a wealth of knowledge to the research on precious fragrance entities. Many other fragrant flowers are used in perfumery (rose, lilac, *Gardenia*, tuberose, *Plumeria*, *Chloranthus,* and *Freesia*).

The methyl jasmonates occur naturally as mixtures of stereoisomers in jasmine essential oil (42) (*Jasminum grandiflorum* and *J. sambac*) and many other flowers (e.g., *Boronia megastigma*, *Osmanthus fragrans, Lonicera japonica,* and *Plumeria alba*). According to Acree et al. (51) the isomer (+)-*epi*-methyl jasmonate (**33**) has an approximately 500 times lower odor threshold than the major isomer (–)-methyl jasmonate (**34**). This could clearly be confirmed after all the stereoisomers were resolved by preparative GC with packed cyclodextrin columns (47). The "sniffing technique" has been an inevitable tool in the evaluation of fragrance and flavor components (52–54). Joulain (81) has pointed out that the composition of the odor bouquet of living flowers and picked flowers may be very different (Figures 13 and 14).

Flower scents from some cactus species were also investigated. Thus, the absolute configuration of (+)-dehydrogeosmin (**35**), a C-12 terpenoid in *Rebutia marsoneri* and *Dolichothele sphaerica* (Cactaceae), could be determined by enantioselective GC and chemical correlation with (–)-geosmin (**36**) of known absolute configuration (82). The same compound was correlated with a new epoxy-trinoreudesmane sesquiterpene (**37**), the odor impact compound of the liverwort *Lophocolea bidentata* (Figure 15) (83).

Lilac alcohols (**38**) and lilac aldehydes (**39**) (a mixture of four possible stereoisomers each) have been identified in *Syringa vulgaris* (lilac) flowers. Only the synthetic material is used in perfumery. The separation of enantiomers by enantioselective GC with a cyclodextrin derivative, the determination of the absolute configuration by asymmetric synthesis, the sensoric profiles of the isomers (84) and studies toward the biosynthetic of these fragrance compounds were reported recently from the research group of Mosandl (85). A very useful compilation of flower scents was published by Bergström et al. (86) covering 118 original articles between 1966 and 1992.

Other fragrance and flavor compounds

Many other chiral fragrance compounds were investigated and resolved by enantioselective GC, and distinct differences in the odor intensity and character were noted. The four stereoisomers

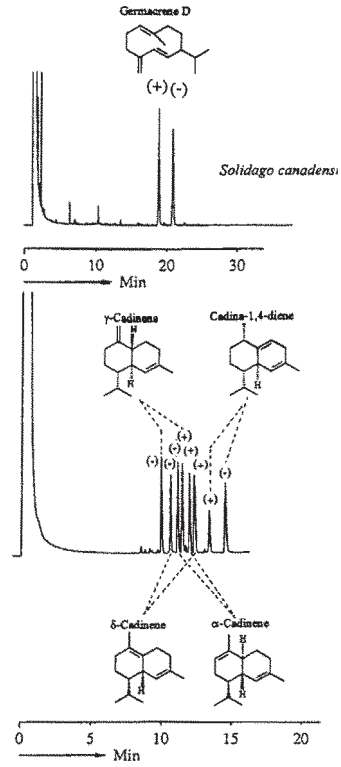

Figure 11. Essential oil of *Solidago canadensis* with both germacrene D (**8**) enantiomers (top) and rearrangement products generated by acidic ion exchange resin (below). For the figure, 25 m heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (50% in OV 1701, w/w) at 100°C.

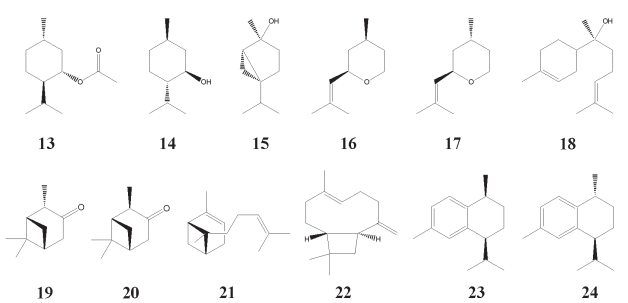

Figure 12. Compounds 13–24.

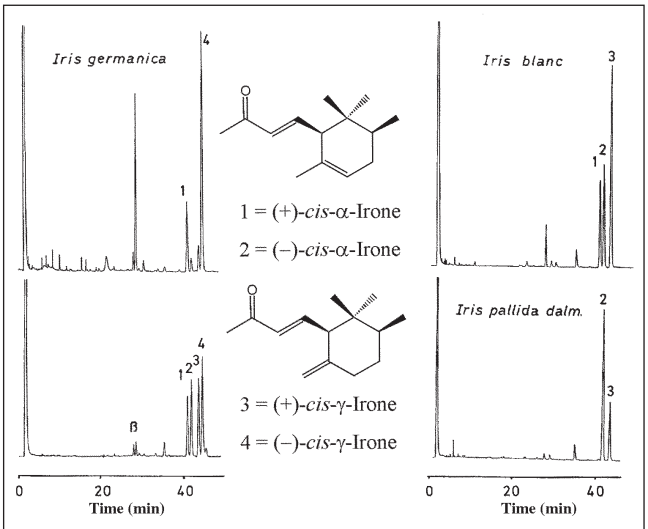

Figure 13. Enantiomer separation of *cis*-α- (**26**) and *cis*-γ-irone (**27**) (lower left side) and determination of the enantiomeric composition of these comounds in several Iris species. For the figure, 25-m capillary with octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin at 105°C.

of nerolidol, a sesquiterpene alcohol present in many essential oils, were first resolved on heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (42) and different sensoric properties were reported for the *E*/*Z*-isomers and their enantiomers (87). The first enantiomer separation of the macrocyclic fragrance compound muscone (3-methylcyclopentadecanone) was also achieved with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (42). (*R*)-(–)-Muscone (**40**), originally isolated from a deer *Moschus moschiferus*, was found to have a very powerful note, whereas the (*S*)-enantiomer was qualified as poor and less strong (88).

Synthetic musk flavor compounds such as tonalide and galaxolide (consisting of four stereoisomers) and their chiral metabolites in the environment could be resolved on heptakis(6-*O*-t.butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin with a remarkable selectivity (Figure 16), and chiral discrimination by living organisms was documented (89). Only two of the four stereoisomers [*trans*-(4*S*,7*S*)-galaxolide (**41**) and *cis*-(4*S*,7*R*)-(–)-galaxolide (**42**)] were reported to have a pleasant musk note, but the other steroisomers were described as almost odorless (90).

A diterpene type compound isolated from sperm whales (*Physeter macrocephalus*), ambrox (**43**), was resolved on heptakis(6-*O*-t.butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (47). In this case, the (–)-enantiomer has a woody, strong, and warm animal note that is lacking in the (+)-enantiomer (91).

Although sulfur compounds are not too common in essential oils, some plants such as *Agathosma betulina* and *A. crenulata* (buchu leaf oils) produce 8-mercapto-*p*-menthan-3-one (**44**) and its S-acetate as powerfully smelling constituents with a characteristic "cassis" flavor. Both compounds were prepared as mixtures of four stereoisomers and separated by enantioselective GC (92). By comparison of the same compounds prepared from enantiomerically pure pulegone, the stereoisomers could be assigned and evaluated with respect to their sensoric properties (93).

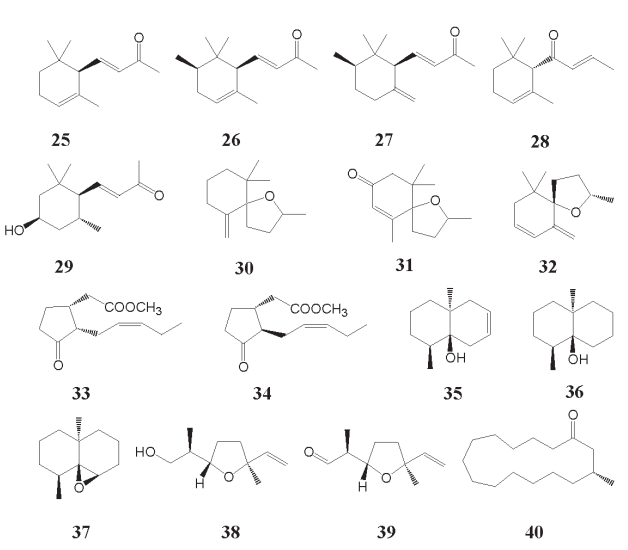

Figure 14. Compounds 25–40.

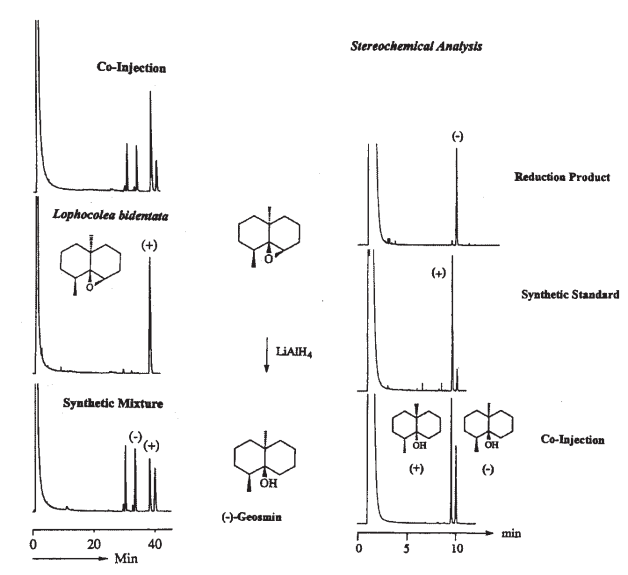

Figure 15. Chemical correlation and enantioselective GC of synthetic and natural trinorsesquiterpene (37) from the liverwort *Lophocolea bidentata* with (–)-geosmin. For the figure, 25-m capillary with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin (20% in OV 1701, w/w) at 120°C.

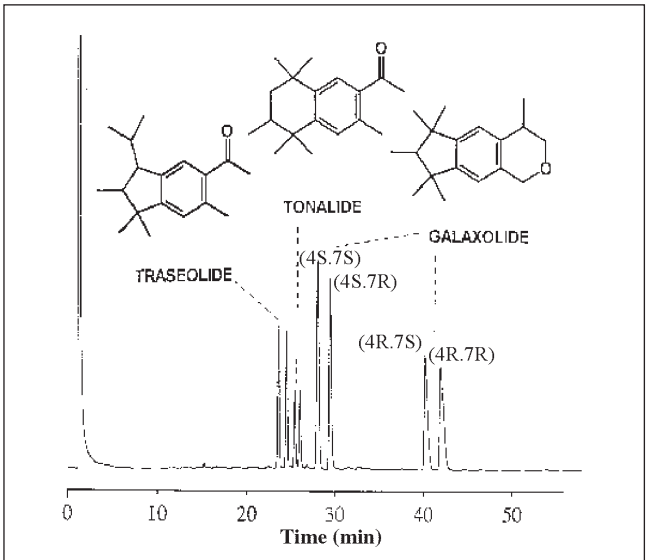

Figure 16. Enantiomer separation of synthetic musk flavor compounds. For the figure, 25-m capillary column with heptakis(2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (50% in OV1701, w/w) at 140°C.

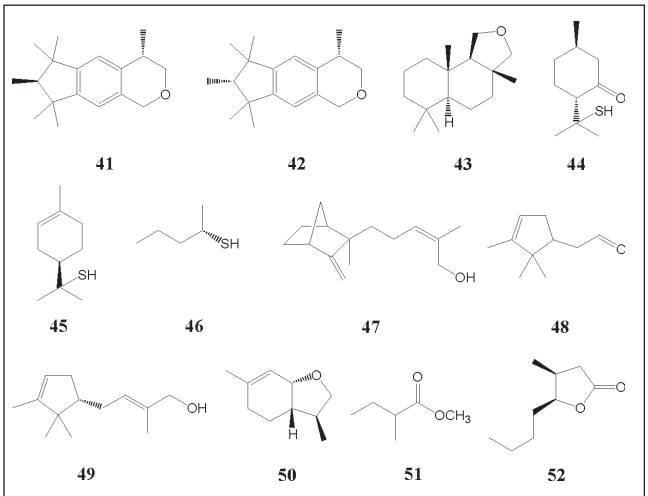

Figure 17. Compounds 41–52.

Another sulfur compound, (+)-(*R*)-1-*p*-menthene-8-thiol (**45**), was originally identified by Demole et al. (94) in grapefruit juice (*Citrus paradisi*). It is considered as the compound with the lowest threshold value of all flavor compounds ever found in nature (odor threshold, 10^{-4} ppb). The (*S*)-(–)-enantiomer was reported to have the more fruity and natural aroma (94). Its enantiomer separation and a new organoleptic investigation favors the (*R*)-enantiomer as the active principle, but the (*S*)-enantiomer has a week and unspecific flavor (95). The "tropical" aroma of the guava fruit (*Psidium guava*) is ascribed to 2-pentanethiol. The resolution of the enantiomers was described by Schreier et al. (96). Only the (*S*)-enantiomer (**46**) was found in guava extracts.

Sandalwood essential oil (*Santalum album)* contains approximately 25% of (–)-(*Z*)-β-santalol (**47**), which is considered as the principal vector of the woody, warm odor of this highly esteemed product (97). Its enantiomer is described as odorless (98). Many synthetic chemicals with sandalwood odor [e.g., α-campholene aldehyde (**48**) and its derivative 2-methyl-4-(2,2,3-trimethylcyclopent-3-en-1-yl)-but-2-enal (Madrol) (**49**)] have been evaluated.

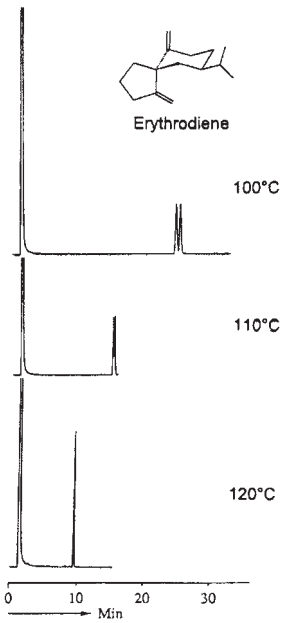

Figure 18. Enantiomer separation of sesquiterpene hydrocarbon erythrodiene (62) on a 25-m capillary column with heptakis-(2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (50% in OV1701, w/w) at 100°C, 110°C and 120°C.

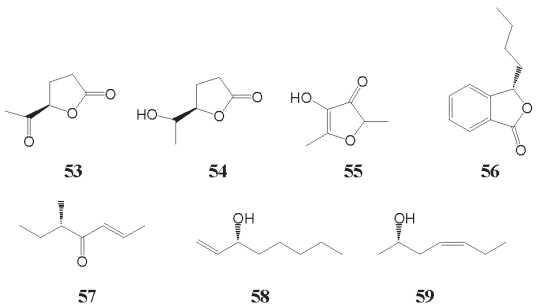

Figure 19. Compound 53–59.

Some of them were investigated by enantioselective GC and the enantiomers (99) exhibited different odor impressions. Only the (–)-(*S*)-enantiomer of Madrol exerts the typical sandalwood odor (100).

Dillether (**50**) is known as the most important chiral flavor component in dill (*Anethum graveolens*). The enantiomer separation was achieved using octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin (101) and the absolute configuration of the natural isomer confirmed in comparison to an enantiomerically pure reference (102).

Plant volatiles from ripe fruits are important vectors of flavor compounds. The highly volatile methyl- (**51**) and ethyl 2-methylbutanoates have repeatedly been investigated by food chemists. In apples and many other fruits, beer, wine, and cheese—almost exclusively—the (*S*)-enantiomers of these chiral esters are present. They differ by their more pleasant odor from their enantiomeric counterparts (103,104).

Fruit flavor is often dominated by the odor of γ- and δ-lactones. They are present in many exotic fruits like passion fruit, mango, papaya, but also in strawberry, apricot, peach, and raspberry. The first efficient and direct enantiomer separation was achieved by König et al. using hexakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-α-cyclodextrin (lipodex B) (105), which nicely resolves the series of γ-lactones. This includes the four stereoisomers of 4-butyl-3-methylbutyrolactone found in whisky, in which the (3*S*,4*S*)-enantiomer (**52**) predominates (106) (Figure 17).

The determination of the enantiomeric composition of γ-lactones is also possible using heptakis(3-*O*-acetyl-2,6-di-*O*-pentyl)-β-cyclodextrin (lipodex D) (107). γ-Lactones in different fruits were investigated and characteristic enantiomeric proportions were detected with this chiral selector (108).

The stereo differentiation of lactones could be extended to d-lactones and many other substrates using octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin (lipodex E) (27), and it was possible for the first time to determine the enantiomeric composition of d-lactones in milk, butter, and coconuts (109). Later g-lactones from fruits, beverages, and food products (110) and d-lactones from fruits and beverages were investigated, and the natural enantiomeric composition was used to detect adulterations with synthetic "nature-identical" (racemic) flavors. Some 5-substituted γ-lactones [(*R*,*S*)-solerone (**53**) and (4*R*,5*R*)/(4*S*,5*S*)-solerole (**54**)] were identified as flavor compounds in sherry wines. Their almost racemic composition is attributed to racemization during storage (111).

Furanones such as furaneol (4-hydroxy-2,5-dimethyl-3-[2H]-furan-3-one) (**55**) are racemic because of their configurational instability (tautomerism). These flavor compounds are found in many fruits and fruit products as pineapple, strawberry, grapes, and the corresponding juices and wines. Their enantiomeric composition was investigated by enantioselective GC (112). 3-Butylphthalide (**56**) is a strong smelling chiral compound present in many Apiaceae, such as celery, and was stereochemically investigated using enantioselective GC . The natural (2*S*)-enantiomer is dominating (95:5) and has a significantly lower odor threshold value than its enantiomer (113).

Other chiral constituents from food products [e.g., filbertone (**57**) from hazelnuts (114) or aliphatic alcohols such as (*R*)-1-octen-3-ol (**58**) ("mushroom flavor") (4) and (*S*)-(*Z*)-4-hepten-2-

ol (**59**) and its acetate ("banana flavor") (115)] have also been analyzed by enantioselective GC.

An excellent reference source for these fragrance and flavor compounds and their analysis by enantioselective GC is the review of Werkhoff et al. (68) in which the enantiomer separations of some rare types of chiral flavor compounds are also documented (Figures 18 and 19).

Enantioselective GC

Technique

GC enantiomer separation dates back to the 1960s when Gil-Av performed his pioneering work with chiral amino acids and peptide derivatives as the first successful chiral selectors (116). This work was continued by several groups and cumulated in the development of "Chirasil-val" by Frank et al. (117). In parallel, several similar chiral selectors as the polysiloxane-derived XE-60-L-val-(S)-phenylethylamide and similar compounds were established and commercialized (118). Although primarily suitable for the analysis of substrates with the ability to form hydrogen bonds to the chiral selector, specific derivatization of hydroxy groups (e.g., with isocyanates to urethanes (119)] provided the first general methods for the resolution of many terpenoic alcohols from essential oils (120). Another reagent, phosgene, could be used for conversion diols to cyclic carbonates (121), and ketones such as fenchone or camphor were derivatized to oximes (122).

It should also be mentioned that an alternate technique, complexation GC, as introduced by Schurig, has been a useful technology in flavor and fragrance analysis (123).

A break-through in the development of chiral selectors for GC was the introduction of cyclodextrin derivatives in the late 1980s. After the report of astonishing results about the discrimination of the enantiomers of monoterpene hydrocarbons by native cyclodextrins dissolved in organic solvents by Koscielski et al. (124) and permethylated cyclodextrins by Szeijtli et al. (125), this lead was eventually taken up by other groups, and permethylated cyclodextrins were investigated more carefully (126). The performance of capillary columns with these compounds having a high melting point was greatly improved by dilution with polysiloxanes by Schurig et al. (5,127). At the same time, the first hydrophobic cyclodextrin derivatives (128,129) with different types of substitution patterns for the 2-, 3-, and 6-position of the cyclodextrin glucose units were prepared, and their utility (even undiluted) and broad potential for enantiomer separation of almost any kind of volatile chiral substrate was realized (4).

The following cyclodextrin derivatives have been mainly applied in essential oil analysis: hexakis(2,3,6-tri-*O*-pentyl)-α-cyclodextrin (lipodex A) (128,130);

heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (127,131); heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (42,132); heptakis(6-*O*-methyl-2,3-di-*O*-pentyl)-β-cyclodextrin (101); heptakis(2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (133); heptakis-(2,3-di-*O*-acetyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (40); heptakis-(3-*O*-acetyl-2,6-di-*O*-pentyl)-β-cyclodextrin (lipodex D) (107); octakis-(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin (lipodex G) (101); octakis-(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin (27); octakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-γ-cyclodextrin (42); and octakis-(2,6-di-*O*-pentyl-3-*O*-trifluoroacetyl)-γ-cyclodextrin (134).

A general guide for the selection of specific cyclodextrin derivatives for a specific separation problem is still hard to provide, but the rule is that polar substrates (i.e., hydroxy compounds) may be better resolved on acylated cyclodextrin derivatives, wheras nonpolar substrates such as hydrocarbons are better separated on per-alkylated cyclodextrin derivatives. The grade of polarity of a column can be determined as documented in the literature (4).

Many applications of some of the cyclodextrin derivatives listed above in flavour and fragrance analysis with corresponding literature references were documented by Schreier et al. (135). Very useful for the less experienced analyst could be the published *Collections of Enantiomer Separation Factors* (136–140).

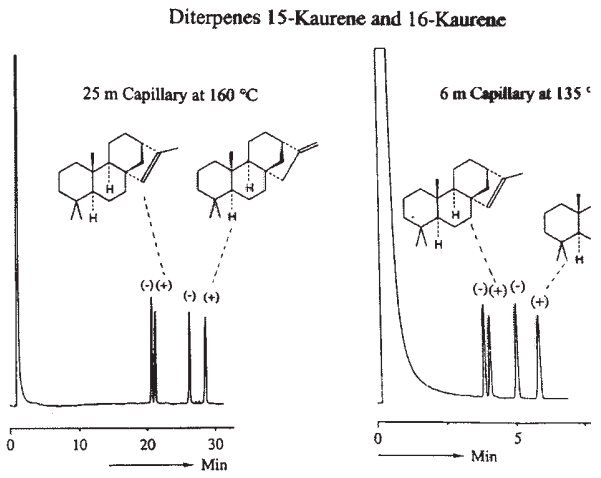

Figure 20. Enantiomer separation of diterpene hydrocarbons 15-kaurene (**60**) and 16-kaurene (**61**) on a 25-m capillary column with heptakis(2,3-di-*O*-methyl-6-*O*-t.butyldimethylsilyl)-β-cyclodextrin (50 % in OV 1701, w/w) at 160°C (left) and on a 6-m capillary with the same stationary phase at 135°C (58).

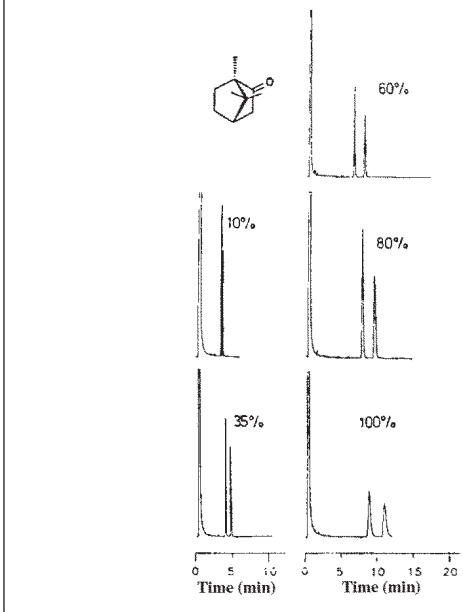

Figure 21. Enantiomer separation of camphor (**63**) on 25-m capillary columns with different percentages of coating with octakis (3-*O*-butyryl-2,6-di-*O* pentyl)-γ-cyclodextrin (154).

Operational parameters

Optimization of the GC parameters is, of course, always beneficial in the performance of analytical GC, but it is absolutely inevitable to achieve optimum enantiomer separations. Most importantly, the column temperature plays a crucial role because thermodynamics of chiral discrimination are ruled by many different parameters (141). Essentially, the resolution is highest at the lowest column temperature. As demonstrated in Figure 18, a temperature that is too high may conceal enantioselectivity completely and only appear after lowering the operational temperature by 20°C. This cannot always be easily adjusted because commercial capillary columns are available at a certain column length. Nevertheless, in certain cases one should consider cutting the columns in pieces and using a shorter piece at a lower temperature to achieve higher resolution factors (α-values). At comparable retention times for baseline separation of racemic diterpene, hydrocarbons 15- (**60**) and 16-kaurene (**61**) will take almost 30 min at a column temperature of 160°C, but only 5 min at 135°C with a 6-m piece of capillary with comparable resolution. (58,141) (Figures 20 and 21).

The dilution of cyclodextrin derivatives in a polysiloxane and the type of polysiloxane are also important factors. Heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin has a very high melting point (~ 200°C) and can be used at dilutions of 10% (maximum 20%) only. Most t.butyldimethylsilyl-substituted cyclodextrin derivatives can be used at a 1:1 dilution (50%, w/w). The lowest operational temperature may then be around 75°C, which could be too high for highly volatile compounds. In this case the use of 20% cyclodextrin derivatives or, even better, chemical immobilization can significantly reduce the lowest operational temperature (142). The most hydrophobic cyclodextrin derivatives can be used in percentages of 60–100%, although no improvement of the resolution is observed above 80% (Figure 21). However, depending on the pre-treatment of the capillary inner surface, the stability of the coating (film stability) may be red (Figure 22).

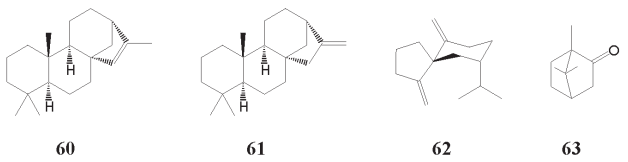

Figure 22. Compounds 60–63.

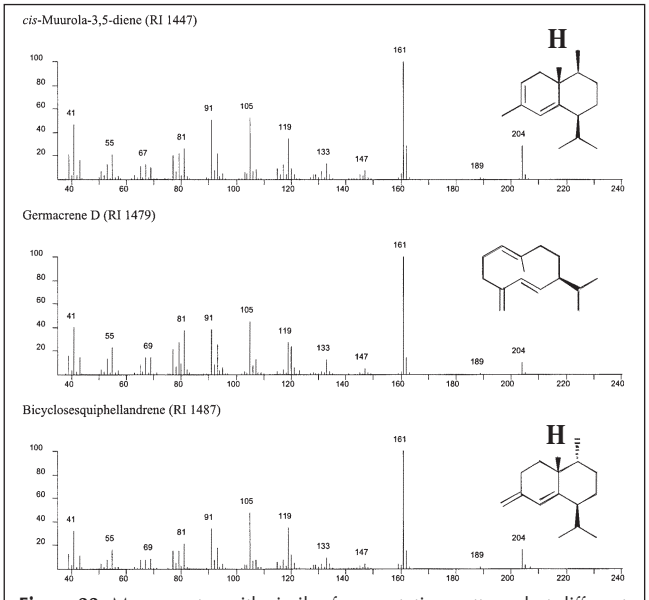

Figure 23. Mass spectra with similar fragmentation patterns but different retention indices.

Identification of essential oil constituents by GC–MS

Identification of known compounds

An essential oil usually contains a large number of constituents of concentrations differing by many orders of magnitude, and it may take considerable time to screen such mixtures for new or unknown natural compounds.

Unfortunately, a high number of publications in the field of terpenoid chemistry does not entirely fulfill expectations in the reliability of constituents reported or compounds claimed to be new. Typical problems in the context of identification and assignment of essential oil constituents by GC–MS methodology are: (*a*) nonrecognition of known compounds, which leads to an elevated number of so-called "unknown constituents", causing wasted research efforts and investments for repeated structure elucidation and unnecessary publications of these compounds wrongly claimed to be new; (*b*) incorrect assignment and mismatching of known compounds, leading to publication of incorrect lists of constituents, which are subsequently very hard to detect and be corrected by the scientific community, and, even worse, may be multiplied by other (less experienced) research groups; and (*c*) incorrect assignment of *unknown* compounds, leading to undiscovered new constituents and again to wrong lists of constituents of the essential oils studied.

There are several issues to be taken into account that could improve the overall quality and reliability of GC–MS-based identifications and assignments of constituents of essential oils:

The popular and huge mass spectral libraries like Wiley or NIST 98 do not cover terpenoid constituents in an extent necessary to claim unidentified spectra as new compounds. The Automated Mass Spectral Deconvolution and Identification system (AMDIS) software is more or less dedicated to the perfumery field (143). Very useful for mass spectral comparison is the data bank established by Adams (144). However, it is not yet supported by an

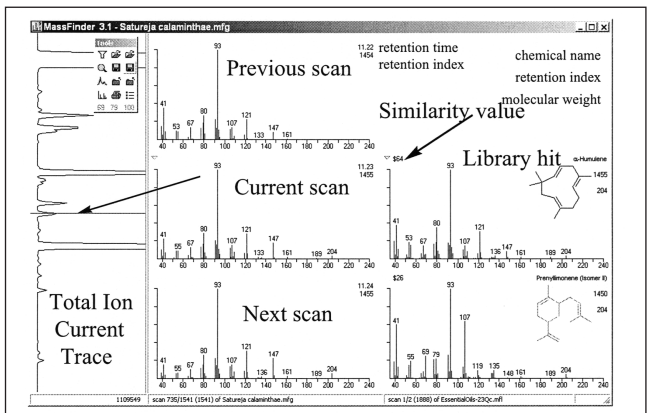

Figure 24. Illustration of the mass spectral identification system "MassFinder". Total ion current trace (left); current scan, as indicated by cursor (center); and library hit for current scan (right part).

interactive software. The majority of sesquiterpenes (145) and diterpenes discovered in the last 5 years are unlikely to be included in general libraries. Thus, specialized mass spectral libraries and current literature have to be employed in order to avoid nonrecognition of previously published compounds. The recently released mass spectral library *Terpenoids and Related Constituents of Essential Oils* may be an answer to this problem (146).

Of highest importance is the proper use of retention indices in addition to the mass spectrum in order to reliably identify constituents. Figure 23 shows three very similar mass spectra that cannot be distinguished unequivocally. However, in combination with accurate retention indices, a reliable assignment of the three compounds is possible beyond doubt.

To stress the previous point, only retention indices (i.e., Kovats index system related to *n*-alkanes), not retention times, should be employed throughout. Although retention times will vary considerably with gas velocity, carrier gas pressure, or slightly differing column lengths, and, most importantly, cannot be compared with retention times measured on other systems, retention indices are constant enough (provided, they are correctly assigned to the correct compounds) to be compared between scientists around the world when using a standard stationary phase such as polydimethylsiloxane (PDMS), which is preferred by many researchers because of its outstanding robustness.

There are computer programs to facilitate the use of retention indices and base library search methods on both dimensions (i.e., retention index and mass spectral similarity), which significantly improves the rate and quality of search hits. A popular program following this philosophy is MassFinder (147), which also allows the convenient and rapid creation of proprietary mass spectral libraries. One needs to only once measure an *n*-alkane pattern spanning the whole retention time range, and the computer will without further efforts handle GC–MS data on a retention index based time scale.

Research groups and commercial companies in the field of essential oils should be equipped with a personal electronic mass spectral library covering all relevant spectra of the essential oils studied, including unknown compounds or preliminary identifications.

Published mass spectra should be free of background noise or contaminating peaks and exhibit enough intensity to properly show all expected isotopic patterns. The publication of mere lists of some of the most abundant peaks of novel compounds is not state-of-the-art anymore and should be replaced by complete lists or graphical representations and, most importantly, supplementary data available online (text lists preferred for general use). Publications should always include retention indices from the most reliable and reproducible PDMS. Columns with this phase are very robust, even when provided from different suppliers. This is not always true for the wax columns (polyethylene glycol), which are still very popular in flavor research because of their greater polarity and selectivity.

The MassFinder software provides transfer of GC–MS data of most used formats, from an MS directly to a computer, and the software compares the data with the mass spectral library and retention indices of plant volatiles obtained under identical instrumental and experimental conditions (same column; same temperature program, magnetic field, or quadrupole type of MS; and same ionization mode). This mass spectral library (~ 2,000 entries) is currently the leading and most up-to-date collection of sesquiterpene hydrocarbons, oxygenated sesquiterpenes, monoterpenes, diterpenes, and natural aliphatic and aromatic constituents commonly found in essential oils, flavors, and fragrances.

Our efforts in maintaining a high reliability when identifying mass spectra and assigning structures have been significantly facilitated by the use of the new version (MassFinder 3), which features some unique methods for handling GC-MS data. Most importantly, MassFinder 3 employs a two-dimensional search algorithm which simultaneously takes mass spectral similarity and retention index into account when looking for the best matching library entry. This drastically increases the search quality and avoids wrong assignments of highly similar mass spectra of different compounds. A unique feature is the inverse search, which allows the determination of whether the mass spectrum of interest occurs anywhere in the given GC–MS run. MassFinder 3 also covers many other aspects chemists need when handling GC–MS data. Although the two-dimensional real-time search is the dominant feature, the convenient and easy-to-learn user interface offers many more sophisticated characteristics. Identified or unknown peaks can be annotated in the chromatogram, and graphics can be exported in publication-ready quality. Flexible filtering/sorting modes (ion traces, base peak filtering, background subtraction, etc.) and very easy library extension and modification further expands the scope of MassFinder 3. Newly identified compounds can be easily included into the library by direct transfer of MS data from a GC–MS run on screen.

The use of MassFinder is illustrated in Figure 24. Although the left part of the screen shows the total ion current trace, a mass spectrum can be selected by the cursor at any scan of the GC–MS run. The right part of the monitor simultaneously shows the best library hit for this mass spectrum together with the structure, scan number, and retention index. This system has proved to be a very reliable tool for rapid, semiautomatic identification of compounds present in the library, and it can be easily adapted to most commercial instruments.

Identification of unknown constituents

Much more effort is necessary for an unambiguous identification of new compounds. Only in rare cases of simple mass spectra can the corresponding structure be derived directly from the mass spectrum and the identification proved by direct comparison with a reference compound, which may be available from the lab or must be prepared by synthesis. The more common procedure is the preparative isolation of a single compound in amounts sufficient for NMR investigations. For this purpose, several chromatographic steps are preformed in a certain order. Usually the process starts with a simple silica column chromatography; by applying the solution of an essential oil on a column with dry silica as proposed by Kubeczka et al. (148). Preparative GC using packed columns, preferentially with cyclodextrin derivatives (63), is efficiently used to collect fractions from an essential oil containing the peaks of interest. If this step does not provide sufficient purity, a thick-film capillary column with greater separation efficiency (again cyclodextrin columns are preferred because of

their extraordinary chemo- and enantioselectivities) is used for further purification. For structure investigations, the whole range of 1- and 2-dimensional NMR techniques can be utilized.

As mentioned before, stereochemistry (relative and absolute configuration) needs to be determined. Although relative configurations in most cases can be derived by the nuclear Overhauser effect or nuclear Overhauser enhancement spectroscopy techniques, absolute configuration needs to be established either by comparison with a synthetic reference compound or by chemical correlation. This means that a new structure may be modified by hydrogenation, oxidation, dehydration, ozonization (149) or rearrangement reaction to a known compound or to a product that is compared with a product derived from a known structure with established absolute configuration by mechanistically known steps. Finally, the products are compared using enantioselective GC. A typical example is shown in Figure 15. More examples are given in the literature (60). Very small sample amounts are usually sufficient for such a procedure, and the results derived from the GC are very conclusive.

Conclusion

Enantioselective analysis of chiral constituents of essential oils and flavor and fragrance compounds has arrived at a point of perfection that is attributable to the work of a number of research groups in different disciplines (natural compound chemists, food chemists, perfumery chemists, and, more recently, molecular biologists). However, the analysis of essential oils and flavor and fragrance compounds is not only a fascinating research field but also of considerable economical and social relevance. New regulations in the European Union (150) on alleged allergenic essential oil constituents forces essential oil dealers and the perfumery industry (151) to adopt more sophisticated analytical procedures including GC–MS and enantioselective GC. In the field of aromatherapy (152), the naturalness of essential oils is very highly ranked and, again, the analysts are to be at stake. The commitments demanded by health authorities concerning declarations of active constituents and of possibly toxic compounds in plant volatiles and phytopharmaceutical formulations also require more research activities. This will also be important in the coming decades, which will confront us with increasing numbers of genetically engineered plants with new fragrance and flavor characteristics. In the past few years, a number of floral scent genes have been isolated from highly fragrant flowers (153), and new flavor genes were isolated from fruit (strawberry) and vegetable tissues (154). For centuries the fragrance of roses has been highly appreciated. Over 18,000 rose cultivars are known, and products obtained from them have an annual value of approximately $10 billion (155). Recent work on the genetic equipment of roses will probably open the door for many new fragrances (156).

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