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Title: Enantioselective gas chromatography with cyclodextrin in odorant analysis

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This chapter concerns enantioselective gas chromatography with cyclodextrin derivatives as chiral stationary phases for chiral recognition of volatile odorants in the flavour and fragrance field. The text is divided into two main parts. The first one is more general and deals with enantiomers and odour and need for chiral recognition, evolution of chiral stationary phases for enantioselective GC since its beginning, followed by the history of cyclodextrins and their applications to enantioselective GC. It also includes some theoretical aspects of enantiomer separation with cyclodextrin derivatives and their influence on routine chiral recognition.

The second part concerns the strategy of chiral recognition in routine analysis with cyclodextrin derivatives as chiral stationary phases illustrated by examples with real natural product samples. This part describes enantiomer automatic identification or their excess or ratio determination in complex mixtures by enantioselective GC combined with mass spectrometry; in particular it deals with the potential of multidimensional techniques and of fast GC in chiral recognition and the role played by mass spectrometry. The last paragraph concerns the use of total analysis systems in chiral recognition.

1. Introduction

Metabolite formation in a biological matrix (plant or animal) is almost always stereo-guided, and the resulting components are very often chiral and present an enantiomeric excess. Enantiomer recognition and enantiomeric excess (ee) and/or ratio (er) determinations of a chiral compound are therefore important parameters to characterize the matrix and its biological activity, in particular in the fields of food, flavour and fragrance. Enantiomeric recognition is therefore very important:

(i) to correlate chemical composition and organoleptic properties;

(ii) to determine the biosynthetic pathway of a compound

(iii) to classify a sample;

(iv) to determine the geographic origin of a “natural” sample;

(v) to implement quality control and detect frauds or adulteration of “natural” samples: enantiomeric composition can reveal the addition to “natural” products of cheap synthetic materials or volatiles from other sources, mainly to reduce costs.

The interaction of a molecule with olfactory receptors is usually stereoselective, meaning that the organoleptic properties of the enantiomers of a chiral molecule can be different and may elicit different odour sensations or intensities. The earliest evidence of this different perception of chiral odorants was found in 1961 by Rienacker and Ohloff [1]; it concerned β-citronellol, whose (+)-β-enantiomer was described as having a typical citronella odour, while that of the (-)-β-enantiomer was rated as a geranium type smell. Since then the different odour responses of a very large number of enantiomeric chiral odorants have been investigated [2,3]; Table 1. reports some examples of chiral compounds whose enantiomers present different odours.

<Table 1 here>

2. Chiral recognition of odorants and enantioselective gas-chromatography (Es-GC)

Gas chromatography (GC), in particular in combination with mass spectrometry (MS), is the most widely used and powerful technique to define the composition of the volatile fraction of natural product samples. However, GC with conventional stationary phases fails with enantiomer recognition because separation is based on physico-chemical properties, whose values are the same for both enantiomers of the chiral molecule. Stationary phases with a chiral selector in their structure that interacts differently with each enantiomer of the chiral

compound(s) must therefore be used to achieve enantiomer separation. The first stationary phase for chiral separation of enantiomers by gas chromatography was introduced by Gil-Av et al. of the Weizmann Institute of Science, Israel; in 1966 [4-6] they separated racemic amino acid alkyl esters on glass capillary columns coated with *N*-trifluoroacetyl-L-isoleucine lauryl ester.

Several chiral selectors have since been proposed, that operate on different principles. Three of these, distinguishable for the mode of selector-selectand interaction, have been successfully applied to routine analysis [7-9]:

- ✓ Separation of enantiomers on chiral amino acids via hydrogen bonding [4-6,10-14];
- ✓ Separation of enantiomers on chiral metal coordination compounds via complexation [15,16];
- ✓ Separation of enantiomers on cyclodextrin derivatives via inclusion (inter alia) [17,18].

Cyclodextrin (CD) derivatives are the chiral stationary phases (CSPs) most widely used in the flavour and fragrance field today, because of their wide range of application and high enantioselectivity, which offer the possibility to separate the underivatized enantiomers of a large number of chiral molecules with different structures and organic functions. The development of stationary phases based on different separation approaches has, however, made available appropriate CSPs for almost all enantiomer separations.

The next paragraphs will briefly discuss the characteristics and chiral separation mechanisms of CSPs operating via hydrogen bonding, complexation with chiral metal coordination compounds, and cyclodextrins, this last in greater detail because of the importance CDs now have in the flavour and fragrance field.

2.1 Chiral stationary phases based on hydrogen bonding

The first chiral stationary phases available were those based on hydrogen bonding. They are usually enantiomerically pure amino acid-derived selectors, mostly applied to separate racemic amino acid derivatives, although they can also be used to separate other chiral molecules.

As already mentioned, separation of the enantiomers of *N*-trifluoroacetyl amino acid alkyl esters was first achieved in 1966, on *N*-trifluoroacetyl-L-isoleucine lauryl ester (**1)** (Figure 1) using a glass capillary column [6] and then, in 1967, on *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester (**2)** (Figure 1) using a packed column [5], in both cases by Gil-Av's group. It was later shown that an additional amide function was fundamental to provide additional

hydrogen bondings. The C-terminal amino acid was therefore replaced by an amine derived from valine, yielding the diamide (**3**) [19], and then coupled to a copolymer of dimethylsiloxane via the amino function, to yield Chirasil-Val (**4**) in both enantiomeric forms [11,12]. Epimeric CSPs, e.g. (L,*R* and L,*S*) (**5**) [20], containing two chiral centres were also introduced, although they can either enhance (matched-case) or compensate for (mismatched-case) enantioselectivity. Koppenhoefer et al. modified the chiral backbone in Chirasil-Val (**4**) by varying loading and polarity, and by introducing rigid spacers [21-23]. Calixarene derivatives (Chirasil-Calix) [24] were also tested, but failed to markedly improve enantioselectivity.

Enantiomer separation by hydrogen-bonding CSPs usually requires the analyte (generally an amino acid) to be derivatized, to increase its volatility and/or to introduce functions suitable for additional hydrogen-bonding association [13].

<Figure 1 here>

2.2. Chiral stationary phases based on metal coordination

Complexation gas chromatography was introduced by Schurig in 1977; he applied a chiral metal coordination compound (dicarbonyl rhodium(I)-3-trifluoroacetyl-(1*R*)-camphorate (Figure 2) dissolved in squalane to separate the enantiomers of the chiral olefin 3-methylcyclopentene [25].

<Figure 2 here>

The chiral recognition effectiveness of these CSPs was confirmed by the enantiomer peak inversion when both enantiomeric selectors obtained from (*1R*)- and (*1S*)-camphor were applied, and by peak coalescence with the racemic selector [26]. Complexation gas chromatography was later extended to chiral oxygen-, nitrogen- and sulfur-containing compounds, using various chiral 1,3-diketonate bis chelates of manganese(II), cobalt(II) and nickel(II) derived from perfluoroacylated terpene-ketones. The main limit of these CSPs was the low operative temperature range (25-120°C); their thermostability was increased when the immobilised polymeric CSPs (Chirasil-Nichel) were introduced [27]. Before the advent of modified cyclodextrins, complexation GC was successfully applied to chiral analysis of volatile non-hydrogen-bonding compounds such as pheromones [28], flavours and fragrances [28,29] and products of enzymatic reactions, such as oxiranes [30].

Enantioselective complexation GC has also been used extensively to study the principles of chiral recognition.

2.3 Chiral stationary phases based on (inter alia) inclusion

The separation of enantiomers based on inclusion, using cyclodextrin derivatives as chiral selector, is probably now the most widely adopted approach for Es-GC analysis, in particular in the flavour and fragrance field.

2.3.1 Cyclodextrin derivatives as chiral selectors - A short history

General overview of cyclodextrin chemistry [31]

The first reference to a substance that later proved to be a cyclodextrin (CD) was published by Villiers in 1891; he isolated a crystalline substance from starch digested from *Bacillus amylobacter* [32,33]. Twelve years later, Schardinger found that small amounts of two different crystalline products were formed when starch was digested by that microorganism [33]. Then, in the 1930s, Pringsheim and his group described the inclusion properties of crystalline dextrins and their acetates [34] and discovered their ability to form complexes with organic compounds having different structures. An important contribution to the elucidation of the crystalline structure of Schardinger's dextrin was given by Freudenberg and co-workers who, later in that decade, hypothesized that cyclodextrins consisted of maltose units linked through α-1,4-glycosidic linkages. In 1936 they postulated the cyclic structure [35]. In the 1950s several groups, in particular those of French [36] and Cramer [37], studied in depth the chemical and physical properties of CDs, at the same time clarifying their enzymatic production. By the late 1960s, these studies led to the full elucidation of cyclodextrins' structures and physical properties, and their ability to form inclusion complexes was demonstrated; methods for their laboratory-scale preparation were also now developed. The three major CDs are crystalline, homogeneous, non-hygroscopic compounds consisting of torus-like macro-rings built up of 6 to 8 glucopyranose units (Figure 3) i.e. the α-CDs, β-CDs and γ-CDs, respectively. The CD ring is a conical cylinder in which the primary hydroxyl groups (position 6) are situated on one of the two edges of the ring and the secondary groups (positions 2 and 3) are placed on the other edge.

<Figure 3 here>

Several thousands of publications have discussed possible applications of CDs: a large proportion (nearly 25%) are in the pharmaceutical field, while about 20% concern applications to analytical chemistry and diagnostic preparations. The analytical applications of CDs mainly refer to their use in GC, HPLC, capillary zone electrophoresis, and, to a lesser extent, thin layer chromatography (TLC), and to enhance of UV-Vis absorption and luminescence/phosphorescence.

Chiral stationary phases based on cyclodextrin derivatives for Es-GC

The first chiral separation in GC with CD as stationary phases was due to Koscielski and Sibilska [38]; in 1983 they separated the enantiomers of α- and β-pinene, Δ-3-carene and hydrogenated derivatives, on columns packed with a mixture of native α-cyclodextrin in formamide. Despite their high separation factor α, these columns had a limited lifetime and low efficiency. CDs were applied to capillary gas chromatography almost contemporarily, by Juvancz et al. in 1987 [39] and Schurig et al. in 1988 [16]. In other pioneering studies, Alexander et al. [40] and, shortly afterwards, Venema and Tolsma [41], demonstrated that undiluted permethylated β-cyclodextrin could be employed in capillary columns for high-resolution separation of enantiomers at high temperatures. Subsequently, two different approaches were introduced to overcome the problems associated with the high melting point of permethylated cyclodextrin:

(a) Konig and co-workers proposed using per-*n*-pentylated cyclodextrins, which are liquid at room temperature. These CD derivatives can thus be used as such to separate enantiomers belonging to several classes of compounds [42-45].

(b) at about the same time, Schurig and Nowotny introduced permethylated-β-cyclodextrin dissolved in moderately polar polysiloxanes (e.g., OV-1701) [16,46]. This approach combined the high enantioselectivity of the modified cyclodextrin with the very good gas chromatographic characteristics of polysiloxanes.

Dilution in polysiloxane is now almost the only approach in routine use. A number of in-depth investigations [47,48] have, in fact, made it possible to obtain chiral columns with chromatographic properties, efficiency and reliability comparable to those of conventional columns, that can operate successfully over an extended range of temperatures (0-240°C).

Chemistry of cyclodextrin derivatives for Es-GC

The presence of three hydroxyl groups that can be regio-selectively alkylated and acylated offers an enormous number of possible α-, β-, γ-cyclodextrin derivatives. Numerous derivatives have been described, mainly based on β-cyclodextrin derivatives, although no universally-applicable derivative has yet been found. A fundamental improvement was the introduction of bulky substituents, e.g., the *tert*-butyldimethylsilyl (TBDMS) or *tert*-hexyldimethylsilyl- (THDMS) groups [49,50] at the primary C6-hydroxy groups, first introduced by Blum and Aichholz [49,51], but mainly developed by Mosandl's group [51]. The bulky substituent conditions the CD conformation and inhibits entrance to the cavity at the smaller rim, thus orienting the analyte/CD interaction towards the wider rim, with the substituents at the C2- and C3-secondary hydroxy groups responsible for enantioselectivity.

The development of new CD derivatives is still necessary, to extend the operative range and to embrace new applications such as multidimensional or fast Es-GC, as well as to increase enantioselectivity in comparison to existing ones. Unfortunately, the number of new CD derivatives with higher, wider or "new" enantioselectivity that have been introduced over the last ten years is very small. The authors have quite recently introduced "fully asymmetrically substituted derivatives" [52]. The already existing CD derivatives present the same "small" substituents (mainly acetyl, methyl or ethyl groups) in the C2- and C3-secondary hydroxy groups, and bulky substituents (i.e. *t*-butyldimethylsilyl-, TBDMS) in the C6- primary hydroxyl groups, and at the same time provide good enantioselectivity and columns with good chromatographic properties. However, their enantioselectivities are very often complementary, therefore CD derivatives with a "mixed" substitution pattern in positions 2 and 3 of the wide rim may provide the desired synergy in enantioselectivity (the term "asymmetric" is here used to indicate the different nature of the substituents in positions 2, 3 and 6). The asymmetrically substituted methyl/ethyl derivatives (i.e. $6^{I\text{-}VII}$-*O*-TBDMS- $3^{I\text{-}VII}$-*O*-ethyl-$2^{I\text{-}VII}$-*O*-methyl / $3^{I\text{-}VII}$-*O*-methyl-$2^{I\text{-}VII}$-*O*-ethyl-β-cyclodextrin) were the most successful, since they were found to be more effective, in terms of both the number of chiral compounds separated into their enantiomers, and the resolution values, than the corresponding symmetrical CDs (i.e. $6^{I\text{-}VII}$-O-TBDMS- $3^{I\text{-}VII}$-O-methyl-$2^{I\text{-}VII}$-O-methyl/$3^{I\text{-}VII}$-O-ethyl-$2^{I\text{-}VII}$-O-ethyl-β-cyclodextrin), proving to be very useful in routine analysis for control of samples containing several chiral markers in a single run.

Several other approaches to improve enantioselectivity and chromatographic results have been attempted:

a) applying two or more chiral selectors in a single column, for example 1) mixing two CDs [53-56] although the synergy in terms of enantioselectivity was not as high as expected; similar unsatisfactory results were obtained when two CD units were condensed; and 2) mixing two structurally different chiral selectors, e.g. amino acid derivatives and cyclodextrin derivatives; these were grafted simultaneously [57], linked to polysiloxane [58] or covalently linked to amino acid derivatives/cyclodextrins [59,60]. These binary phases retained the enantioselective properties of each component, thus extending the range of enantioseparation achievable.

b) cyclodextrin selector(s) chemically and permanently linked to a polysiloxane backbone [61-63] (Chirasil-Dex) as a way of advancing the dilution approach.

However, in the authors' opinion great efforts must yet be made to develop a further generation of CD derivatives with extended enantioselectivity and better chromatographic properties

Mechanism of chiral recognition of cyclodextrin based CSP in ES-GC

It is difficult to rationalise chiral recognition involving modified cyclodextrins, since almost all classes of chiral compounds, ranging from apolar to highly polar, are susceptible to being separated into their enantiomers on the appropriate cyclodextrin-derived CSP; there is often no logical dependence on molecular shape, size or functionalities of either selectand or selector. This is because the recognition process is multimodal and may, inter alia, involve inclusion, hydrogen-bonding, dispersion forces, dipole–dipole interactions, electrostatic interactions, and hydrophobic interactions [9,64-66]. The separation of enantiomers is due to the differences between the diastereomeric CD selector–selectand (enantiomer) association equilibria; although this is a low-energy interaction, separation is achieved, partly thanks to the high efficiency of capillary GC columns [13]. Lipkowitz et al. [67-69] made an important contribution to the molecular modelling of enantiomer separation with cyclodextrins, demonstrating the dichotomy that exists between the location of the preferred binding site of a selectand within the cavity, and that of the optimum chiral discrimination domain, which may, a priori, be different; they also discussed the importance of short-range dispersion forces as intermolecular forces. Another significant contribution, from Schurig's group, showed that inclusion of a molecule within the CD cavity may not be a prerequisite for a successful chiral recognition; they reported enantiomer separations obtained with per-*n*-pentylated amylose [70] and with modified linear dextrins [71,72].

All these considerations combine to demonstrate that a strong molecular association is not always a prerequisite to efficient chiral discrimination [64] and that a weak selectand–selector interaction can often lead to successful chiral recognition. These results also explain possible changes of elution order of enantiomers from members of homologous series [73] and the unreliability of correlating absolute configuration with elution order.

Thermodynamics of enantioseparation by gas chromatography

Schurig and coworkers introduced a thermodynamic model to describe enantiomer separation with CD derivatives [18,74]. The model is based on two equilibria, that condition the elution of a volatile solute, *B,* eluting from a column containing a solution of a cyclodextrin derivative, *A,* in an achiral solvent, *S,* as stationary phase; K_L^0 is the partition coefficient of *B* between the gas (*g*) and the liquid (*l*) phases, and *K* is the thermodynamic stability constant of the complex *AB* in the achiral solvent.

(i) $B_{(g)} \overset{K_L^0}{\longleftrightarrow} B_{(l)}$

(ii) $B_{(l)} + A \overset{K}{\longleftrightarrow} AB$

K_L^0 in the two equilibria can be defined through the activities (*a*) of the reagents in the solvent, i.e.

$$K_L^0 = \frac{a_{B_{(l)}}}{a_{B_{(g)}}} \qquad \text{for equilibrium (i)} \qquad (1)$$

and

$$K_L^0 = \frac{a_{AB}}{a_A a_{B_{(l)}}} \qquad \text{for equilibrium (ii)} \qquad (2)$$

Since the total amount of solute *B* in the liquid phase is $a_{B_{(l)}} + a_{AB}$, the apparent partition coefficient K_L is

$$K_L = \frac{a_{B_{(l)}} + a_{AB}}{a_{B_{(g)}}} \qquad (3)$$

that, from equations (1) and (2), can be rewritten as [75,76]

$$K_L = K_L^0(1 + Ka_A) \qquad (4)$$

On the basis of the fundamental equation of chromatography

$$t' = \frac{K_L}{\beta} t'_m \qquad (5)$$

where t'_m is the dead time, *t'* the net retention time, K_L the partition coefficient between mobile and stationary phase and β the phase ratio; an analogous equation can be obtained for the net retention time

$$t' = t'_0 (1 + Ka_A) \quad (6)$$

where t_0 is the net retention time on an identical reference column, containing only the solvent *S* as stationary phase.

Equation (6) affords a definition of the retention increase (or retention increment) *R'* [77] as

$$R' = Ka_A \quad (7)$$

as the quantitative measure of the increase in the retention of *B* due to the cyclodextrin derivative, *A,* diluted into the achiral solvent, S.

Equation (6) must be rewritten in terms of relative retention data to obtain an equation that is independent of column length, diameter and film thickness, since experimentally it is impossible to obtain truly identical columns to determine *t'* and t'_0 [77]:

$$r' = r_0(1 + R') \quad (8a)$$

or

$$R' = \frac{r'}{r_0} - 1 \quad (8b)$$

where *r'* is the relative retention of solute *B* calculated *versus* an inert reference standard *B**:

$$r = \frac{t'}{t'^*} \quad \text{[chiral column (A in S)]} \quad (9)$$

$$r_0 = \frac{t'_0}{t'_0{}^*} \quad \text{[achiral reference column (only S)]} \quad (10)$$

If solute *B* is a racemic mixture of the enantiomers B_R and B_S, and *R* arbitrarily represents the last-eluted enantiomer, since $K^0_{LR} = K^0_{LS}$, enantiomer separation of B_R and B_S must be due to different values of K_R and K_S. The chiral separation factor is therefore given by

$$\alpha = \frac{K_{LR}}{K_{LS}} = \frac{t'_R}{t'_S} = \frac{r_R}{r_S} \quad (11)$$

Equation (11) can be modified on the basis of equations (4) and (7):

$$\alpha = \frac{K_R a_A + 1}{K_S a_A + 1} = \frac{R'_R + 1}{R'_S + 1} \quad (12)$$

From the thermodynamic relationship of free enthalpy

$$\Delta G = -RT \ln K \tag{13}$$

and the ratio of the thermodynamic stability constants

$$\frac{K_R}{K_S} = \frac{R'_R}{R'_S} = \frac{r_R - r_0}{r_S - r_0} \tag{14}$$

it is possible to determine the difference in the free enthalpies of formation of the diastereomeric associates:

$$\Delta_{R,S}(\Delta G) = \Delta G_R - \Delta G_S = -RT \ln \frac{K_R}{K_S} = -RT \ln \frac{R'_R}{R'_S} \tag{15}$$

and the enthalpic and entropic contributions to $\Delta_{R,S}(\Delta G)$, from the Gibbs-Helmotz equation:

$$\Delta_{R,S}(\Delta G) = \Delta_{R,S}(\Delta H) - T\Delta_{R,S}(\Delta S) \tag{16}$$

This equation also indicates that, for a 1:1 molecular association, the quantities $\Delta_{R,S}(\Delta S)$ and $\Delta_{R,S}(\Delta H)$ display an opposite effect $\Delta_{R,S}(\Delta G)$. At the isoenantioselective temperature T_{iso}:

$$T_{iso} = \frac{\Delta_{R,S}(\Delta H)}{\Delta_{R,S}(\Delta S)} \tag{17}$$

a peak coalescence occurs [$-\Delta_{R,S}(\Delta G)=0$, $K_R = K_S$, no separation of enantiomers]. Above $T_{iso,}$ *the* enantioselectivity ($\Delta_{R,S}(\Delta G)$) changes and is governed by $\Delta_{R,S}(\Delta S)$, while below T_{iso}, it depends on $\Delta_{R,S}(\Delta H)$.

The most important impact of these results on routine Es-GC is that, in most cases, even at high temperatures, enantioselectivity is dominated by enthalpy-control, with an increase in the separation factor α with decreasing temperature. As a consequence, the lowest possible temperature for Es-GC separation of enantiomers must be applied, not least because the separation of enantiomers by gas chromatography with CD derivatives as chiral selector is based on fast kinetics and is thermodynamically driven [78].

3. Measurement of the enantiomeric distribution

The main aim of the GC separation of enantiomers is to precisely determine the enantiomer distribution of one (or more) marker components in a matrix, in order to characterize it. This aim is further stimulated by the legislation on chiral compounds, which is becoming increasingly stringent, thus requiring that reliable methods for enantiomer composition determination be developed.

Enantiomeric distribution is usually expressed in terms of *enantiomeric excess* (*ee*), *enantiomeric composition* (*ec*) or *enantiomeric ratio* (*er*) [79]. *Enantiomeric excess* expresses the superabundance of one enantiomer over the other, and is defined as

$$ee = \frac{E_1 - E_2}{E_1 + E_2} \tag{18}$$

where E_1 and E_2 are the areas of the enantiomers, E_1 being the major enantiomer; *ee* ranges from 0 for racemic mixture to 1 for pure E_1. In routine practice, *ee* is often expressed as a percentage

$$\%ee = \frac{E_1 - E_2}{E_1 + E_2} \cdot 100 = \%E_1 - \%E_2 \tag{19}$$

Enantiomeric purity has also been used as a synonym for *ee*.

Enantiomeric composition (ec) is defined as the molar fraction of the major enantiomer x_{E_1} in a mixture:

$$ec = x_{E_1} = \frac{E_1}{E_1 + E_2} \tag{20}$$

In this case too, in routine, *ec* is in general expressed as a percentage

$$ec\% = x_{E_1} = \frac{E_1}{E_1 + E_2} \cdot 100 \tag{21}$$

Lastly, the *enantiomeric ratio*, *er*, is defined as

$$er = \frac{E_1}{E_2} \tag{22}$$

where E_1 is the major enantiomer; *er* extends from *ee* = 1, for a racemic mixture, to *ee* = ∞, for pure E_1. The terms *er* and *ee* are correlated as follows

$$ee = \frac{(er - 1)}{(er + 1)} \tag{23}$$

and

$$er = \frac{(1 + ee)}{(1 - ee)} \tag{24}$$

In routine analysis, correct measurement of the above parameters requires that the peaks of the two enantiomers be baseline separated, i.e. that their resolution is $R_S \geq 1.5$. The separation factor α cannot therefore be used routinely in the flavour and fragrance field, because it does not consider peak widths, and must be determined isothermally.

4 Enantioselective GC analysis with cyclodextrins in the flavour and fragrance field

Enantiomer separation by Es-GC with columns coated with modified cyclodextrins as chiral selector has been successfully applied to several fields of contemporary research, such as checking essential oil authenticity [80], flavours and fragrances and alcoholic beverages [81,82], and clinical chemistry [82]. Terpenoids [83], enzymatic reactions [84], organochlorine pesticides [85,86], alkyl nitrates as atmospheric constituents [87] and volatile pharmaceutical compounds can also be investigated [64].

The second part of this chapter will be devoted entirely to chiral recognition strategies in the flavour and fragrance field by Es-GC, with CD derivatives as stationary phase; it is based on the authors' day-to-day experience in this field.

Cyclodextrin derivatives are the most widely used chiral selectors in Es-GC of chiral components in the flavour and fragrance field, most probably because their physico-chemical characteristics (polarity, reactivity and volatility) are highly "compatible" with the enantiomer discriminative interactions provided by CDs.

As is the case of all stationary phases, CDs have advantages and disadvantages, both absolutely and in comparison to the other CSPs described above; their main advantages are:

- they can separate underivatized enantiomers, therefore enabling real natural product samples to be directly analyzed without any manipulation;
- they can separate almost all classes of volatile chiral compounds, thanks to the wide range of selectivity covered by the large number of available CD derivatives;
- they possess good chromatographic properties (efficiency and inertness) and a wide range of operative temperatures of columns prepared with CDs, thanks to their dilution in moderately-polar polysiloxanes.

However, CDs also present some disadvantages, limiting not only their popularity but also the routine use of Es-GC, because they tend erroneously to be considered complex:

- absence of a "universal" cyclodextrin derivative separating most chiral compounds; this means that a laboratory must be provided with a number columns to extend the range of analysable chiral compounds;
- difficulty of identifying enantiomers and measuring *ee* and/or *er* in real-world complex samples with mono-dimensional Es-GC, because of the increased probability of interferences due to the doubling of the number of chiral analyte peaks; this limit is extremely frequent in the flavour and fragrance field;

- long analysis time, due to the use of long columns and low temperature rates, because of the cyclodextrin mechanism of separation.

Partly in view of the above three points, the next paragraphs will examine the optimization of strategies for the chiral recognition of odorous markers by Es-GC and Es-GC-MS, with CD as chiral selectors in complex mixtures in the flavour and fragrance field, based mainly on the authors' experience.

5 Testing column efficiency and enantioselectivity.

A short paragraph must first be dedicated to a critical and often-neglected point: column efficiency and enantioselectivity. Conventional GC columns in general, but even more so Es-GC columns, must periodically be tested for their efficiency and enantioselectivity, in order to obtain reliable results. This evaluation requires the use of dedicated test mixtures, such as the Grob test to evaluate chromatographic performance of a column over time, and a chiral test for enantioselectivity. In the authors' laboratory, a test mixture containing racemates with different volatilities, structures and polarities has been designed to check column enantioselectivity; it is used continually [47] (its composition is reported in the caption of Figure 4). Figure 4 reports the chiral test profiles carried out on the four columns used to build the chiral library (see paragraph 6.2).

<Figure 4 here>

6 Analysis of enantiomers in complex samples

Reliable chiral recognition of marker compounds in complex real-world samples generally requires a two-dimensional approach, in order to avoid erroneous identifications due to peak overlapping; this is a particular risk because of the doubling of the number of peaks of chiral analytes when separated by Es-GC. Two complementary but distinct approaches are therefore available: the first is to adopt *a second dimension in separation* (by conventional heart-cut GC-GC or comprehensive GC) (see paragraph 6.1), while the second approach implies using a *second dimension in detection,* by coupling GC with mass spectrometry (MS). The latter method applies a strategy that is the converse of the conventional one, i.e. the enantiomer(s) is(are) located in the chromatogram by their MS spectrum and identified by GC data (i.e Kovats retention indices (*I*s) [88], or linear retention indices (I^Ts)) [89,90]. This is because, as is known, MS is not a chiral probe, so that the mass spectra of two

enantiomers cannot be distinguished. This makes it impossible to determine which enantiomer is present in a sample. Conversely, chromatographic data (and in particular linear retention indices) are the most reliable and effective parameters for enantiomer identification, being characteristic for each analyte (enantiomer), since they depend on its chromatographic interaction with the adopted stationary phase; similar considerations can be made with locked retention times, which were introduced by Giarrocco et al. in 1997, and by Blumberg and Klee in 1998 [91,92] (see below).

6.1 Location and identification of enantiomers in complex samples by multidimensional gas-chromatography (i.e. with a second dimension in separation)

The basic concepts of *multidimensional* gas-chromatography are described in a dedicated chapter of this book, and will therefore not be discussed here. Two techniques commonly known as multidimensional GC are at present used in chiral recognition:

i) heart-cut GC-GC is as yet the most widely used approach for *ee* and *er* determination of chiral compounds in complex mixtures, in particular for applications in which a small number of chiral components (or fractions) (possibly eluting at relatively different temperatures) are to be submitted to chiral recognition. With this technique, *the selected chiral components* (peaks) eluting from the first, achiral, column (1st dimension -^{1}D) coated with a conventional stationary phase are on-line and automatically transferred to the second, chiral, column (2nd dimension - ^{2}D), through a dedicated time-programmable interface, for a programmed time fraction of the whole chromatographic run [93-95]. Figure 5 reports the HS-SPME heart-cut GC-GC profile of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway *HS-SPME* with an HP-5 (25m x 0.25mm d_c x 0.25mm d_f) as first column and 30% 6$^{I\text{-}VII}$-*O*-TBDMS-2$^{I\text{-}VII}$-3$^{I\text{-}VII}$-*O*-ethyl-β-CD in PS086 (25m x 0.25mm d_c x 0.25mm d_f mm) as second column; for analysis conditions and peak identification see Figure caption.

<Figure 5 here>

ii) two-dimensional comprehensive GC (GC×GC) is suitable for highly complex mixtures, in which several components are to be submitted to chiral recognition. In GC×GC *each component* eluting from the first column is on-line and automatically trapped, refocused, and re-injected into the second column, in a fixed time (4-8 s) by a thermal or valve-based focusing device (modulator) [96]. Unlike heart-cut GC-GC, the GC×GC system for chiral

recognition consists of a chiral column of conventional i.d. and length (^{1}D) connected to a very short narrow-bore column (^{2}D) coated with a conventional stationary phase, to enable fast analyses to be run during the time of one modulation. The chiral column must be in the first dimension, because of the high efficiency required for effective enantiomer separation [97,98]. Figure 6 reports the GC×GC contour plots of lavender essential oil (for analysis conditions see Figure caption).

<Figure 6 here>

6.2 Location and identification of enantiomers in complex samples by gas chromatography and mass spectrometry (i.e. with a second dimension in detection)

As already mentioned, enantiomers have indistinguishable mass spectra, making chromatographic data indispensable for their reliable identification in chiral recognition. The most widely used and reliable methods to identify sample components through gas chromatographic data are based on retention indices (I^Ts) or, alternatively, on the retention time locking (RTL) approach. Either of these systems makes identification more reliable than the simpler retention time or relative retention time approaches, since they overcome most of their limits, in terms of precision and repeatability. However, correct identification of an analyte in a sample, through chromatographic data, always implies the availability of pure reference standards; this is particularly significant in the case of Es-GC in which, to establish the correct order of elution of each enantiomer within a pair, it is indispensable to have a single enantiomer standard, or one or more real-world samples in which one precisely identified member of the pair is present as such, or in a well-known enantiomeric excess.

Retention indices were first introduced by Kováts [88] for isothermal analysis (Is); their use was later extended to temperature programmed analysis (Linear Retention Indices (I^Ts)) by Van den Dool *et al.* [89,90]. I^T is a number that expresses the entity of the analyte/stationary phase interaction relative to a reference standard mixture (homologous series of hydrocarbons or fatty acid methyl or ethyl esters) and provides its unequivocal position in the chromatogram, provided that rigorously standardized analysis conditions are applied. In agreement with the authors' experience, I^Ts with the same stationary phases are indispensable in Es-GC to identify an enantiomer in a sample. This approach implies that the enantiomer is located in the chromatogram through its mass spectrum, and then identified through its I^T by comparison to that of the reference standard determined on the same column. I^Ts in essential oil analysis were reviewed by d'Acampora Zellner *et al.* in

2008 [99] and their importance in plant volatile fraction discussed by Rubiolo et al. in 2010 [100].

In general, the identification potential of GC is somewhat neglected, because of the power of mass spectrometry when used as detector for GC. Few GC–MS software packages therefore include I^Ts information to help component identification, while some of them only report I^Ts values in the library as "blind or inactive" data, making them only useful for additional confirmation [101-107]. On the contrary, the "interactive" use of I^Ts values (i.e. their use as an active identification parameter) can actually be highly effective, since it provides a second orthogonal tool to identify a compound, operating in parallel to MS spectra.

An MS library specific for the identification of enantiomer components in the flavour and fragrance field was developed by the authors, using I^Ts values "interactively" in parallel to MS spectra [108]. The library was built at an interlaboratory level with the collaboration of two research groups, to increase result reliability. It is based on the interactive I^T/mass spectrum system [103] developed by Costa *et al.* [109] for the flavour and fragrance field, where I^Ts are automatically calculated, and incorporated as an active part of the matching criteria together with mass spectra. The correct identification of an analyte is assured by the range within which its I^T must fall (Retention Index Allowance (RIA) see below), which must be determined preliminarily.

The library consists of 134 racemates whose I^T values were determined on four columns coated with different CD chiral selectors. Table 2 reports the list of racemates included in the first version of the library. Four cyclodextrin derivatives diluted at 30% in PS-086 had to be used to obtain the separation of most of the racemates usually analysed in the flavour and fragrance field, in particular:

$6^{I\text{-}VII}$-O-methyl-$3^{I\text{-}VII}$-O-pentyl-$2^{I\text{-}VII}$-O-methyl-β-cyclodextrin (2,6DM3PEN-β-CD) [110, 111]

$6^{I\text{-}VII}$-O-TBDMS-$3^{I\text{-}VII}$-O-methyl-$2^{I\text{-}VII}$-O-methyl -β-cyclodextrin (2,3DM6TBDMS-β-CD) [112]

$6^{I\text{-}VII}$-O-TBDMS-$3^{I\text{-}VII}$-O-ethyl-$2^{I\text{-}VII}$-O-ethyl-β-cyclodextrin (2,3DE6TBDMS-β-CD) [113]

$6^{I\text{-}VII}$-O-TBDMS-$3^{I\text{-}VII}$-O-acetyl-$2^{I\text{-}VII}$-O-acetyl-β-cyclodextrin (2,3DA6TBDMS-β-CD) [112]

<Table 2 here>

The determination of the RIA window, i.e. the range within which the I^T of an analyte has to fall to be correctly identified, is a key point for univocal identification. An effectively operating library should be based on a single RIA window, to be applied automatically to all enantiomers, analysed on all columns investigated. The ideal RIA should be “narrow” enough to include only one of the two enantiomers, but at the same time “wide” enough to avoid the I^T of a given analyte falling outside the range because of retention variation. A reliable RIA therefore requires baseline enantiomer separation and highly stable I^T values, the latter being obtainable, among others, by highly standardized analysis, constantly-tested inert columns, and corrected injection volumes.

The single RIA for this library was obtained from the average RIA of each class of compounds, in its turn determined from the individual RIA of each of the enantiomers of the 134 racemates investigated, analysed on the four enantioselective columns adopted. A single average RIA value of −1 and +2 for all analytes analysed on all the columns investigated was adopted.

AMDIS, (Automatic Mass Spectral Deconvolution) [101] is another software package, developed by the National Institute of Standards and Technology (USA), that actively uses I^Ts, often in combination with NIST Mass Spectral Libraries.

Retention time locking (RTL) [91,92] is a different approach for reliably identifying an analyte from its GC retention data in programmed temperature analysis. The principle underlying RTL involves determining the adjustment of inlet pressure necessary to achieve the desired match in retention time(s) of an analyte(s) with similarly configured GC systems.

6.3 Techniques in which mass spectrometry increases the reliability of chiral recognition

Clearly, the recent advances in mass spectrometry can dramatically increase the diagnostic power of GC-MS, thanks to the routine use of tandem mass spectrometry or MS/MS (mass spectrometry/mass spectrometry or MS^n). These systems consist not only of the last generation of well-established triple quadrupole (QqQ - tandem-in-space) and ion trap (IT-MS - tandem-in-time) MS/MS analyzers, but also include the most recent hybrid mass spectrometers that are based on high-resolution TOF-MS analyzers, as such or as a second MS unit in combination with a quadrupole or an ion-trap analyzer (Q-TOF or IT-TOF, respectively). The resulting hybrid system, in combination with soft ionization ion sources, thus combines the advantages of tandem MS with those of high resolution analysers; these advantages include, for instance, the determination of the exact mass of molecular ions

and/or fragments, at an accuracy below 5 ppm. However, Es-GC alone is sometimes not sufficient to detect the authenticity of a sample, in particular when racemates naturally occur, or when they derive from processing and/or storage or, especially, from adulteration with a synthetic enantiomer. A decisive step towards achieving a unequivocal definition of sample authenticity is combining Es-GC (or even better Es-GC-GC) with isotope ratio mass spectrometry (IRMS). The effectiveness of Es-GC-IRMS is based on the consideration that the ratio between stable isotopes of enantiomers from the same natural source may be expected to be the same (δ-C^{13}) even when a partial enantiomer racemisation has occurred, since both enantiomers are generally formed through the same biosynthetic pathway in the same natural organism. In a GC-IRMS system, analytes eluting from the GC column are on-line combusted to CO_2 in a dedicated oven, from where the combusted product is directly introduced into an IRMS system. The abundances of 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$, $^{12}C^{16}O^{17}O_2$) and 46 ($^{12}C^{16}O^{18}O$) ions in the nmole range are then simultaneously measured with high precision (≤ 0.3 $^{0}/_{00}$), and the peak areas ratio of the two isotopic peaks compared to a standard reference value. A detailed discussion of this technique is outside the scope of this chapter [114].

7 Fast enantioselective GC analysis with cyclodextrins as chiral stationary phase

A further important limit often conditioning the routine use of Es-GC is the long analysis time, due to the small difference in the energy of association between each selectand (enantiomer) and the CD chiral selectors; this means that very high chromatographic efficiency is required to obtain enantiomer separation. As shown in the above paragraphs, Es-GC separation of enantiomers with CDs as chiral selectors is based on fast kinetics, and is entirely governed by thermodynamics; as a consequence, it closely depends on temperature. Long analysis times are therefore to be expected, since long columns and low temperature rates must in general be applied.

Routine applications require the development of Fast Es-GC methods, in order to satisfy the large number of control analyses required. Routine Fast-GC can in general be obtained by acting on column length, inner diameter, and/or flow-rate, and has resulted in the adoption of narrow bore (NB) columns [115]. In fast-Es-GC, narrow bore columns not only increase analysis speed and analyte detectability, because of peak sharpening [116], but also reduce the enantiomer elution temperature; this results in a gain of enantioselectivity that compensates (in full or in part) for the loss of efficiency (N) due to column shortening. Enantiomer separations with CDs with short columns were already under study in the early

1990s; they afford separation even in a few seconds [117-119]. Schurig and Czesla [120] studied the basis for speeding up ES-GC in depth, and concluded that short conventional 0.25mm d_c columns would have to be used for fast Es-GC, because of their good loadability, integration characteristics, use of conventional instrumentation, and lower consumption of carrier gas. However, conventional d_c short columns can only be used successfully for monodimensional fast Es-GC when chiral compounds have to be recognized in low complexity samples and/or when a limited number of enantiomers are to be analysed simultaneously. With medium-to-high complexity samples, as is often the case in the flavour and fragrance field, a highly efficient separation system combined with single- or multiple-ion monitoring-MS detection (SIM-MS or MIM-MS) is necessary to determine *ee* and/or *er* correctly (see paragraph 3). This paragraph critically discusses the two complementary methods developed and adopted in the authors' laboratory to speed up routine Es-GC analyses [121,122]. The two methods will now be briefly described and illustrated through the analysis of a lavender essential oil. Lavender essential oil is used as an example for both approaches, because it is characterized by several chiral markers whose enantiomeric composition is reliably described in the literature [123,124]. Lavender essential oil contains several optically-active components: α- and β-pinene, camphene, β-phellandrene, limonene, 1-octen-3-ol, camphor, linalool, borneol, linalyl acetate, terpinen-4-ol, lavandulol, α-terpineol and lavandulyl acetate.

MS plays a crucial role in the first approach [122]. This consists of searching for the best trade-off between speed of analysis and loss of resolution of chiral compounds, even at the expenses of separating the other sample components. This is valid since the separated enantiomers can be highlighted by operating in extract-ion, SIM- or MIM-MS modes. Analysis time can therefore be reduced by exploiting the excess of resolution that columns coated with the last-generation of CD derivatives can provide for several chiral compounds, by acting on column dimension, flow rate and temperature rate. The essential requisite is to maintain baseline separation of the enantiomers of the chiral compound(s) investigated, so as to afford correct ee or er determination. With this approach, co-elution of the chiral components with other sample components, due to column shortening and increased heating rates, is to be expected but should not interfere with enantiomeric recognition of the chiral marker(s), because MS as a second dimension in detection, in extract ion mode (or SIM- or MIM-MS) with diagnostic and specific ions, reliably discriminates them from other co-eluting peaks.

Figure 7 reports the Es-GC–MS profiles of the lavender essential oil analysed with the reference conventional column, together with the identification of peaks of chiral components.

<Figure 7 here>

Table 3 compares enantiomer resolutions of chiral components, analysis time, and % analysis time reduction, when lavender essential oil was analysed with the reference conventional column, and with the 10, 5 and 2 m narrow bore columns. Column length and analysis conditions were considered adoptable for routine analysis only when enantiomer resolutions of all chiral compounds were above 1.5.

<Table 3 here>

The results show that: 1) the 10 m NB column can only be used at 2°C/min, because at higher rates terpinen-4-ol, linalyl acetate and borneol are not base-line separated, 2): the highest temperature rate for the 5 m NB column was 5°C/min, because at 10°C/min resolution of terpinen-4-ol was only 1.2, as is clear from its ion profiles at m/z 71, 111, 154; 3) similar considerations can be made for 2m NB columns. Under these conditions, the analysis time was reduced from about 40 min with the reference column to about 14 min with the 5 m NB column. Figure 8 reports the Es-GC–MS profiles of the lavender essential oil analysed with the 5m NB column at 5 (A) and 10°C/min (B) and the extract ion profiles of terpinen-4-ol (71,111,154 m/z) at 5 (C) and 10 °C/min (D).

<Figure 8 here>

The second approach [121] is based on the opposite strategy, i.e. shortening analysis time by seeking the maximum separation efficiency of the chromatographic system, by optimizing analysis conditions. The routine analyses of a large number of different samples in a single field (e.g. aromas from different matrices) are in general carried out under the same standardized GC conditions, partly because of the possibility of automatically identifying peaks from chromatographic data (relative retention times, linear retention indices, retention time locking etc.). Usually, satisfactory separations are obtained under non-specific routine conditions, thanks to much-higher-than-required efficiency of the

chromatographic system, to the detriment of analysis times. Optimization of analysis conditions for a specific sample, i.e. tuning a dedicated method for each matrix, can successfully and dramatically speed up routine GC analyses. Blumberg and co-workers [125-129] investigated in depth the most important theoretical concepts required to optimize capillary GC methods and to obtain the best speed/separation trade-off, while preserving separation and peak elution order.

These studies resulted in the well-known GC method-translation [130], a short description of which will now be given. This approach implies optimizing the chromatographic conditions to give the best speed/separation trade-off with a conventional d_c column, and then transfering the optimised method to a shorter narrow-bore column. The parameters influencing a GC analysis are divided into two main groups: *translatable* (i.e. column dimension (d_c and length), outlet pressure (1 atm for FID, vacuum for MS, etc.), carrier gas and flow rate) and *non-translatable* (i.e. stationary phase type and phase ratio). The crucial operative parameter is hold-up time, which is taken as time unit to express all time-related parameters (duration of temperature plateau(s) and heating rate(s)) and to determine the normalized temperature programme. Two methods are translatable only when non-translatable parameters and normalized temperature programmes are identical. The method-translation principles applied to a given temperature-programmed analysis enables either the flow rate to be optimized, producing the highest efficiency (i.e. the plate number) of a given column (efficiency-optimized flow, EOF), or a combination of flow rate, column dimensions and carrier gas to be determined that corresponds to the shortest analysis time for a given required plate number (speed-optimized flow, SOF [127]). Method translation software can be downloaded for free from the Internet [130].

Optimization of Es-GC analysis conditions of lavender essential oil with a conventional 25 m × 0.25 mm column consisted of three main steps a) choice of initial conditions to be optimized, b) determination of optimal multi-rate temperature program for a predetermined fixed column pressure, and c) determination of optimal pressure (i.e. flow-rate) for the normalized optimal multi-rate temperature program.

The lavender essential oil was first analysed with the conventional d_c column, under the temperature and flow conditions applied to routine analyses, i.e. helium flow rate 1 mL/min and 2°C/min heating rate. Under these conditions, the chiral markers were well separated in an analysis time of 35.2 minutes. Table 4 reports order of elution, retention times (t_R) and resolutions (R_S) of the enantiomers of the chiral markers investigated. Figure 9a reports the Es-GC pattern of the lavender essential oil investigated, analysed under routine analysis

conditions. This CD column and these conditions produced baseline separation of all chiral compounds, with the exception of α-pinene (1) enantiomers, (R_S around 1) and of 1-octen-3-ol (6) enantiomers (not separated at all), while the (*S*)-enantiomers of camphor (7), lavandulol (13) and lavandulyl acetate (12) were not detectable, and (*R*)-lavandulol (13b) and (*R*)-lavandulyl acetate (12b) coeluted. As the starting point for method optimisation, the initial flow rate was doubled to 2 mL/min (4) to reduce the time needed for method development, because this choice did not affect the final optimal conditions.

The optimal multi-rate temperature program at a fixed initial flow-rate was obtained by applying a set of different single-ramp heating rates, namely 2.6, 3.3 and 5.0°C/min (°C/t_M). (see Table 4). The most satisfactory separation was obtained at 2.6°C/min rate, which resulted in an analysis time of about 25.5 min. These experiments showed that, besides the separation of the enantiomers of chiral-markers, lavender essential oil presented three critical pairs of components: α-pinene (1)/camphene (2), 1-octen-3ol (6)/γ-terpinene, and (*R*)-lavandulol (13b)/(*R*)-lavandulyl acetate (12b), that are separated at different heating rates (2.6, 3.3 and 2.6°C/min, respectively). A multi ramp temperature programme was therefore applied to obtain the best resolution of critical pairs in the shortest time; in particular: from 50 to 74°C (elution temperature of (*R*)-α-pinene (1b), retention time 8.62 min) at 2.6°C/min, then to 115°C (elution temperature of (*R*)-lavandulol (13b), retention time 21.79) at 3.3°C/min, then to 220°C at 15°C/min to clear the column. Figure 9b reports the Es-GC pattern of the lavender essential oil analysed under the optimized multi-rate temperature program.

<Table 4 here>
<Figure 9 here>

The next step was to optimize the flow rate by determining the initial EOF (initial flow that maximizes column efficiency and peak resolution) and calculating the initial SOF (initial flow that minimizes analysis time at fixed efficiency) [125]. A reasonable compromise to optimize the flow-rate is to apply the initial value that is optimal for the most critical pair (or the most important pair, in this case α-pinene). This can be EOF, which causes the highest column efficiency for a given solute pair and, as a result, its highest resolution (see below), but it can also be the initial SOF, which causes the shortest analysis time for a given resolution of the critical pair. Ten different pressures were applied to the column, resulting in different initial flow rates. The GC method-translator was used to translate the temperature program

for each pressure, in order to maintain the same normalized temperature program in all cases. Table 5 reports the initial flow rates, the corresponding translated temperature programs, and the resulting analysis times. SOF can be calculated from EOF as SOF = √2 EOF [127], i.e. in this study, the initial EOF was 1 mL/min, so that the initial SOF was 1.4 mL/min, and the corresponding first two heating rates were 2.02 and 2.57°C/min (Table 5). Under these conditions, the analysis time was 29.3 min.

<Table 5 here>

Table 6 reports retention times, enantiomer resolution, and σ values of chiral markers of the lavender essential oil analysed, under the optimal conditions determined. The lavender essential oil profiles at EOF and SOF are shown in Figure 9c and 9d, respectively.

<Table 6 here>

The optimised SOF method with a conventional (25 m × 0.25 mm × 0.25 μm) column was then translated to the corresponding NB column (11.13 m × 0.1 mm × 0.1 μm). Parameters of the translated method are shown in Table 5, and the essential oil profile in Figure 10a. The flow rate was reduced in proportion with the column d_c, i.e. from 1.4 mL/min to 0.56 mL/min, thus assuring SOF operation of the NB column. Under these conditions, and because both columns had very similar length/d_c ratio, translation did not affect resolution of any peak pair (Table 6), but analysis time was reduced to about a third (i.e. 29.50 versus 10.78 min), without loss of peak resolution.

The SOF analysis conditions with ES-GC-FID were then translated to the Es-GC-MS for the same lavender essential oil analysis. Translation from conventional column at SOF to the MS method with NB column at SOF reduced analysis time overall to about a third (retention time of the last peak was reduced from 29.28 min to 10.09 min).

<Figure 10 here>

8 Total Analysis systems and real-world sample analysis

The above paragraphs show that Es-GC is very successful as a technique for chiral odour recognition in the flavour and fragrance field, in particular when considered within the modern strategies of analysis based on fully-automatic systems, better known as “Total

Analysis Systems" (TAS); these are systems in which the three main steps of the analytical process (sample preparation, analysis, and data processing) are on-line integrated into a single step [131,132]. The success of TAS systems is one of the factors that has greatly contributed to the radical change of strategies that began in the early 1990s in analysing volatiles in the flavour and fragrance fields [133].

The adoption of these systems in general, and in particular in chiral recognition, has been made possible by the parallel improvements achieved not only in Es-GC but also in sample preparation and mass spectrometry as detector (see paragraph 6.3). In spite of the number of effective distillation and extraction techniques introduced into sample preparation over this period, the volatile nature of most odorants in the flavour and fragrance field makes headspace sampling, when applicable, the technique of choice for these analyses [100,134,135]. HS techniques have the advantages of being solvent-free, fast, simple, reliable, and, above all, easy to automate; they can also be combined on-line to GC-MS systems. These considerations include both conventional (static (S-HS) or dynamic (D-HS) modes, and High Concentration Capacity Headspace Techniques (HCC-HS) [134-136] HCC-HS are recently-developed techniques that act as a "bridge" between S-HS and D-HS techniques: volatiles are statically or dynamically accumulated on polymers, operating in sorption and/or adsorption modes or, less frequently, on solvents (HS-SPME, HSSE, HS-STE, HS-LPME, etc.). The development of these techniques was favoured by the success of polydimethylsiloxane (PDMS) as an accumulation material for volatiles [137].

Figure 11 reports an example of a TAS where the genuineness of a peach juice is determined by a one-step analysis by HS-SPME-Fast-GC-MS and (SIM-MS) [81]

<Figure 11 here>

9 Conclusions

Cyclodextrin derivatives are nowadays the most effective chiral stationary phases available for Es-GC in the flavour and fragrance field. This consideration is not only justified by the number of CD derivatives with high enantioselectivity and good chromatographic properties offering the possibility to separate most enantiomer pairs without derivatization to the corresponding diastereoisomers, but also to the performance of the resulting GC columns also thanks to the dilution in moderately polar polysiloxanes, in particular: i) high stability and separation repeatability maintained for hundredth of injections, ii) efficiency and analysis times comparable to those of conventional columns, iii) high inertness with classes of

compounds within a relatively wide range of polarity, and iv) extended operative temperature range (20-250°C). As a result, very few enantiomer pairs in this field cannot be separated with these chiral selectors provided that a suitable derivative is applied.

However, in view of an ever increasing demand of chiral recognition, a lot of work has still to be done mainly on two complementary directions: a) the first one concerns an ever better understanding of the mechanisms which is at the basis of enantiomer recognition with CD derivatives in vapour phase thus allowing the operator to design a separation and not to proceed by trial and error, as it is often the case with new enantiomer pairs, and b) the introduction of a new generation of CD derivatives with a more universal enantioselectivity enabling to extend their use to the separation of highly polar chiral compounds.

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Captions to Figures

Figure 1 - Hydrogen-bonding type chiral stationary phases *:* (1) *N*-trifluoroacetyl-L-isoleucine lauryl ester; (2) *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester; (3) N-Lauroyl-L-valyl-tert-butylamide; (4) L-valine-t-butylamide coupled to a copolymer of dimethylsiloxane; (5) L-valine-t-butylamide grafted on to modified polycyanopropylmethyl phenylmethyl silicone (OV-225).

Figure 2 - Coordination type stationary phases: dicarbonyl rhodium(I)-3-trifluoroacetyl-(1*R*)-camphorate

Figure 3 - Structure of α-, β- and γ-cyclodextrines

Figure 4 - Chiral test profiles carried out on the four columns used to build the chiral library (see paragraph 6.2). Peak identification: 1: limonene, 2: 2-octanol, 3: camphor, 4: isobornyl acetate, 5: linalyl acetate, 6: 2-methyl-(3Z)-hexenyl butyrate, 7: menthol, 8: hydroxycitronellal, 9: γ-decalactone, 10: δ-decalactone; a: (R) enantiomer, b: (S) enantiomer, x and y: enantiomer configuration not assigned. *Analysis conditions*: Injection mode: split; split ratio: 1:50. temperature: 230 °C, Det.: FID, temperature 250°C;Temp. programme, 50°C/2°C/min/250°C; (***COPYRIGHT PERMISSION***)

Figure 5 - HS-SPME heart-cut GC-GC analysis of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway*. HS-SPME sampling:* fibre: 2 cm Stableflex 50/30 µm DVB-Carboxen-PDMS (Supelco, Bellafonte, USA); sample amount: 20 mg, vial volume: 20 mL; sampling time: 10 min, temperature: 50°C. *Instrumentation*: Shimadzu QP2010 GC-MS system, provided with a Shimadzu AOC 5000 autosampler with SPME option, and a Shimadzu GC-MS Solution 2.51 software. *Analysis conditions*: Injection mode: split; split ratio: 1:50. Inj. temperature: 230 °C, transfer line: 250 °C; ion source: 200 °C; ionization mode: EI at 70 eV. Scan range: 35–350 m/z. 1st column: HP-5 (25m, d_c: 0.25mm,, d_f: 0.25µm), 2nd column: 6$^{I\text{-}VII}$-*O*-TBDMS-2$^{I\text{-}VII}$-3$^{I\text{-}VII}$-*O*-ethyl-β-CD in PS086 (25m, d_c: 0.25mm, d_f: 0.15 µm). Temp. programme, 1st column 40°C (1min)/3°C/min/250°C; 2nd column, 60°C/2°C/min/180°C. *Peak identification* 1. α-pinene 2.α-thujene, 3. β-pinene 4. sabinene, 5.δ-3-carene, 6. α-phellandrene+myrcene 7. α-phellandrene, 8. limonene 9.β-phellandrene, 10. γ-terpinene, 11. β-ocimene, 12. p-cymene, 13. α-terpinolene, 14. terpinen-4-ol acetate , 15. γ-muurolene, 16.germacrene B, 17.myrtenyl acetate. a: enantiomers (+), b: enantiomers (-).

Figure 6 GC×GC contour plot of lavender essential oil (*Lavandula angustifolia* P. Mill.). Analysis conditions: GC×GC-MS system: Agilent 6890 GC - Agilent 5975 MSD ionization mode: EI 70 eV (Agilent, Little Falls, DE, USA); transfer line temp.: 280°C, scan range: m/z 35–250 in fast scanning mode (12,500 amu/s). GC×GC interface: KT 2004 loop modulator (Zoex Corporation, Houston, TX, USA), modulation time: 4 s. Column set: 1D: 30% $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-ethyl-β-CD in PS086 (25m, d_c: 0.25mm, d_f: 0.15 μm), 2D OV1701 column (1 m, d_c: 0.10mm, d_f: 0.10 μm) (MEGA - Legnano (Milan)-Italy). Modulation loop dimensions: 1 m, d_c : 0.1 mm. Analysis conditions: injection mode: split, ratio: 1/20, temp.: 250°C; carrier gas: helium; Temperature program: 50°C (1 min)/2°C/min/230°C (5 min). Peak identification: 1: 1,8-cineol, 2: β-phellandrene, 3: limonene, 4: 1-octen-3-ol, 5: camphor, 6: linalool, 7: borneol, 8: linalyl acetate, 9: 4-terpineol, 10: lavandulyl acetate; a: *(S)* enantiomer, b: *(R)* enantiomer

Figure 7 - Es-GC–MS profile of the lavender essential oil analysed with the reference column, 30% $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-ethyl-β-CD in PS086 (25m×0.25mm d_c, 0.25 mm d_f). For analysis conditions see text and Table 3. Peak identification: 1: α-pinene, 2: camphene, 3: β-pinene, 4: β-phellandrene, 5: limonene, 6: 1-octen-3-ol, 7: camphor, 8: linalool, 9: borneol, 10: linalyl acetate, 11: 4-terpineol, 12: lavandulol, 13: α-terpineol, 14: lavandulyl acetate; a: (*R*) enantiomer, b: (*S*) enantiomer. 6: not separated; 7a, 12a and 14a: not detected. **(*COPYRIGHT PERMISSION*)**

Figure 8 - Es-GC–MS profiles of the lavender essential oil analysed with the 5m NB column at 5 (A) and 10 ◦C/min (B). Extract ion profiles of terpinen-4-ol (71,111,154 m/z) at 5 (C) and 10 °C/min (D). For analysis conditions see text and Table 3. For peak identification, see caption to Fig. 7. **(*COPYRIGHT PERMISSION*)**

Figure 9 - Es-GC-MS profile of the lavender essential oil analysed under different conditions with a conventional d_c column. For analysis conditions see text and Table 5. For peak identification, see caption to Fig. 7. **(*COPYRIGHT PERMISSION*)**

Figure10 - FID and MS Es-GC profiles of the lavender essential oil analysed under SOF conditions with the narrow-bore column. For analysis conditions see text and Table 5. For peak identification see caption of Fig. 7. **(*COPYRIGHT PERMISSION*)**

Figure 11 - HS-SPME-Fast-Es-GC-SIM-MS profiles of peach juice HS (_____) and γ-C6-C12 and δ-C6-C12 (- - -) standard solutions on a 30 % $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-acetyl-β-CD in PS086 (5m, d_c: 0.10mm, d_f: 0.10 mm. *Sampling conditions*: fibre: 2 cm Stableflex 50/30 μm DVB-Carboxen-PDMS (Supelco, Bellafonte, USA); sample amount:

20 mg, vial volume: 20 mL; sampling time:20min, temperature: 60°C. *Analysis conditions*: Injection mode: split; split ratio: 1:50. Inj. temperature: 230 °C, transfer line: 250 °C; ion source: 200 °C; ionization mode: EI at 70 eV. Scan range: 35–350 m/z. Temp. programme, 90°C/24.0/140°C/60.0/220°C. Peak identification: 1: γ-hexalactone, 2: γ-heptalactone, 3: γ-octalactone, 4: γ-nonalactone, 5: γ-decalactone, 6: γ-undecalactone, 7: γ-dodecalactone, 8: δ-hexalactone; 9: δ-octalactone, 10: δ-nonalactone, 11: δ-decalactone, 12: δ-undecalactone, 13: δ-dodecalactone; a: *(R)*-enantiomer, b: *(S)*-enantiomer.

Table 1- Examples of different odour characteristics of enantiomers of chiral molecules [3]

Compound	Enantiomer	Characteristics
Nootkatone	(5*R*, 6*S*, 8*R*)-(+)	grapefruit
	(5*S*, 6*R*, 8*S*)-(-)	woody-spicy
2-Methylbutanoic acid	(*R*)-(-)	penetrating, reminiscent of cheese and sweet
	(*S*)-(+)	pleasant, sweet, elegant, fruity note
Ethyl 2-methylbutanoate	(*R*)-(-)	first medical, phenolic note, later sweet, fruity
	(*S*)-(+)	ether-like, sweet, after dilution pleasant apple note
2-Ethylhexanoic acid	(*R*)-(-)	herbaceus, earthy
	(*S*)-(+)	sweet, herbaceus, faint musty
1-Octen-3-ol	(*R*)-(-)	intensive mushroom note, fruity, soft
	(*S*)-(+)	herbaceus, green, musty
Linalool	(*R*)-(-)	flower-fresh, reminiscent of lily of the valley
	(*S*)-(+)	differs slightly in odor
(*E*)-Nerolidol	(*R*)-(-)	pleasant, woody, warm, musty
	(*S*)-(+)	slightly sweet, mild, soft, flowery different to (*Z*), less intensive
(*Z*)-Nerolidol	(*R*)-(-)	intensive, flowery, sweet, fresh
	(*S*)-(+)	woody, green, fresh bark
Limonene	(*R*)-(+)	fresh, pleasant, orange-like
	(*S*)-(-)	faint mint note, turpentine note
α–Ionone	(*R*)-(+)	fine violet-like, fruity, flowery, raspberry-like
	(*S*)-(-)	strong woody aspects, raspberry-like
α–Terpineol	(*R*)-(+)	strong, flowery sweet, lilac
	(*S*)-(-)	tarry, reminiscent of colp pipe
Carvone	(*R*)-(-)	herbaceus odor, reminiscent of dill seeds
	(*S*)-(+)	herbaceus odor, reminiscent of spearmint
α–Phellandrene	(*R*)-(-)	citrus odor, slight peppery note
	(*S*)-(+)	weed-like, dill-like
Menthol	(1*R*, 3*R*, 4*S*)-(-)	refreshing, mint note, cool
	(1*S*, 3*S*, 4*R*)-(+)	mint, phenolic note, medical note, camphor-like, not refreshing
Whiskey lactone	(3*R*, 4*S*)-(-)	strong coconut note, reminiscent of celery
	(3*S*, 4*R*)-(+)	piquant celery note, faint coconut note, green walnut note
	(3*R*, 4*R*)(+)	sweet woody, bright fresh coconut note
	(3*S*, 4*S*)-(-)	faint coconut note, faint musty, earthy, reminiscent of hay
δ–Decalactone	(*R*)-(+)	sweet, fruity, milk note
	(*S*)-(-)	sweet, fruity, peach note, fatty, butter-like
Theaspirane	(2*R*, 5*S*)-(-)	highly attractive intense fresh-fruity
	(2*S*, 5*R*)-(+)	naphtalene-like
	(2*R*, 5*R*)-(+)	weak camphoraceus note
	(2*S*, 5*S*)-(-)	fresh camphoraceus note
2-Methyl-4-propyl-1,3-oxathiane	(2*R*, 4*S*)-(-)	sulfurous, herbaceous-green, roasty, linseed oil-like, onion
	(2*S*, 4*R*)-(+)	sulfurous, fatty, friuty-green- tropical fruit, grapefruits
	(2*R*, 4*R*)-(-)	green grass soot, earthy, red radish note
	(2*S*, 4*S*)-(+)	sulfurous, slight bloomy-sweet
γ–Pentalactone	(*R*)	faint, sweet
	(S)	nearly odourless
γ–Hexalactone	(*R*)	faint, sweet coconut with a fatty-herbaceous hay note
	(S)	sweet, creamy coconut with some woody aspects
	(*R*)	sweet, spicy, herbaceous hay note, reminiscent of coumarin

γ–Heptalactone	(S)	fatty, coconut note with fruity-sweet aspects, less intense than the opposite enantiomer
γ–Octalactone	(*R*)	spicy-green, coconut note, with almond notes
	(S)	fatty, coconut note, less intense than the opposite enantiomer
γ–Nonalactone	(*R*)	strong, sweet, soft coconut with fatty-milky aspects
	(S)	fatty, mouldy, weak coconut less intense than the opposite enantiomer
γ–Decalactone	(*R*)	strong, fatty-sweet fruity note, some reminiscence to coconut, caramel
	(S)	soft, sweet coconut note with fruity-fatty aspects
γ–Undecalactone	(*R*)	strong, fatty-sweet, reminiscent of peach, with some bloomy aspects
	(S)	fatty-sweet aldehyde note, less intense than the opposite enantiomer
γ–Dodecalactone	(*R*)	strong, fatty-sweet, bloomy note with aldehyde and woody aspects
	(S)	fatty-fruity, milky notes, less intense than the opposite enantiomer
2-Pentanol	(*R*)	light, seedy, sharp
	(S)	heavy, wild berry, ripe, dusty, astringent
2-Hexanol	(*R*)	mushroom, dusty, oily
	(S)	mushroom, green, ripe, berry, astringent, metallic
2-Heptanol	(*R*)	fruity, sweet, oily, fatty
	(S)	mushroom, oily, fatty, blue cheese, mouldy
2-Octanol	(*R*)	creamy, cucumber, fatty, sour
	(S)	mushroom, oily, fatty, creamy, grape
2-Pentyl acetate	(*R*)	fruity, muscat, green, metallic, chemical
	(S)	fruity, apple, plum, metallic
2-Hexyl acetate	(*R*)	sour, fruity, cherry, plum, strawberry
	(S)	sweaty, sour, fruity, plum, nectarine
2-Heptyl acetate	(*R*)	green, fatty, banana, methyl ketone
	(S)	mushroom, earthy, wild berry
2-Octyl acetate	(*R*)	fatty burnt, boiled vegetable
	(S)	fruity, plum, dusty

Table 2: list of compounds included in the library [108]

Hydrocarbons
α–Phellandrene
α–Pinene
β–Citronellene
β–Citronellene
β–Phellandrene
β–Pinene
Camphene
Caryophyllene
Limonene
Sabinene

Heterocyles
Ambroxide
Menthofuran
Rose oxide

Esters
α–Terpinyl acetate
Bornyl acetate
Bornyl isovalerate
Butyl butyrolactate
cis-2-Methyl-3-hexenylbutyrate
cis -Carvyl acetate
Dihydrocarvyl acetate
Dimethyl methylsuccinate
Ethyl 2-methylbutyrate
Ethyl 2-phenylbutyrate
Ethyl 3-hydroxybutyrate
Ethyl 3-hydroxyhexanoate
Ethyl 3-methyl-3-phenylglicidate
Isobornyl acetate
Isobornyl isobutyrate
Lavandulyl acetate
Linalyl acetate
Linalyl cinnamate
Linalyl propionate
Menthyl acetate
Methyl 3-hydroxyhexanoate
Methyl dihydrofarnesoate
Neomenthyl acetate
Nopyl acetate
Propylene glycolbutyrate
Styrallyl acetate

Lactones
Aerangis lactone
3-Methyl–γ–decalactone
δ–Decalactone
δ–Dodecalactone
δ–Heptalactone
δ–Hexalactone
δ–Nonalactone
δ–Octalactone
δ–Undecalactone
ε–Decalactone
ε–Dodecalactone
γ–Decalactone
γ–Dodecalactone
γ–Heptalactone
γ–Hexalactone
γ–Nonalactone
γ–Octalactone
γ–Pentadecalactone
γ–Pentalactone
γ–Tetradecalactone
γ–Undecalactone
Massoia decalactone
Massoia dodecalactone
Whiskey lactone

Ketones
1,8-Epoxy p-menthan-3-one
3,6-Dimethylocta 2-en-6-one
3-Methylcyclohexanone
3-Oxocineole
α–Damascone
α–Ionone
β–Irone
Camphor
Camphorquinone
Carvone
Fenchone
Isomenthone
Menthone
Methylcyclopentenolone
Nootkatone
Piperitone
Pulegone
Verbenone

Aldehydes
Citronellal
Cyclamen aldehyde
Hydroxycitronellal
Myrtenal
Perillyl aldehyde

Alcohol
α–Bisabolol
1-Octen-3-ol
1-Phenyl ethanol
1-Phenyl-1-propanol
1-Phenyl-2-pentanol
2-Butanol
2-Heptanol
2-Hexanol
2-Methylbutanol
2-Octanol
2-Pentanol
2-Phenyl-1-propanol
3-Hexanol
3-Octanol
4-Methyl-1-phenylpentanol
6-Methyl-5-hepten-2-ol
α–Terpineol
Borneol
cis- Myrtanol
Citronellol
Fenchyl alcohol
Geosmin
Isoborneol
Isomenthol
Isopinocampheol
Isopulegol
Lavandulol
Linalool
Linalool oxide
Menthol
Neoisomenthol
Neomenthol
Nerolidol
Octan-1,3-diol
Patchouli alcohol
Perillyl alcohol
Terpinen-4-ol
Tetrahydrolinalool
trans- Myrtanol
Viridiflorol

Acids
Citronellic acid
2-Methylbutyric acid
2-Phenylpropionic acid
Chrysanthemic acid

Table 3.Enantiomer resolutions of chiral components, analysis time and % analysis time reduction and separation measure *S* obtained for a lavender essential oil analysed with the 10, 5 and 2 m NB columns in comparison with those of the reference column (Ref.).

		Ref.	10 m NB				5 m NB				2 m NB			
Temperature rate (°C/min)		2	2	3.5	5	10	2	3.5	5	10	2	3.5	5	10
Analysis time (min)		40.67	29.35	19.60	14.77	8.67	26.81	17.77	13.60	8.01	28.83	16.57	12.73	7.56
% Analysis time reduction			27.8	51.8	63.7	78.7	34.1	56.3	66.6	80.3	29.1	59.2	68.7	81.4
	$I^{T\,a}$	Resolution												
α–Pinene (1)	*(R)*921/*(S)*923	1.2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Camphene (2)	(-)917/(+)923	6.8	2.2	1.9	1.8	1.7	6.7	5.6	5.6	4.9	3.9	3.5	3.4	3.5
β–Pinene (3)	(+)944/(-)955	5.0	1.8	1.5	1.4	1.2	5.3	4.8	4.4	3.5	3.3	3.3	2.9	2.6
β–Phellandrene (4)	(-)1049/(+)1060	6.1	2.1	1.8	1.7	1.5	6.2	5.3	5.1	4.5	3.1	2.8	2.6	2.4
Limonene (5)	*(S)*1056/*(R)*1072	9.1	2.8	2.5	1.9	1.9	9.0	8.2	8.0	5.0	5.4	4.2	3.8	2.9
1-Octen-3-ol (6)	1126	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Camphor (7)	*(S)*1133/*(R)*1141	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E
Linalool (8)	*(R)*1174/*(S)*1189	6.1	2.6	2.4	2.3	1.9	3.1	3.3	3.2	2.5	3.1	3.4	3.0	3.0
Borneol (9)	*(S)*1192/*(R)*1200	3.0	1.6	1.2	1.2	1.0	2.9	2.5	2.0	1.9	2.1	2.0	1.7	1.6
Linalyl acetate (10)	*(R)*1231/*(S)*1237	3.2	2.6	2.5	1.6	NS	3.8	3.0	2.8	2.4	4.1	3.8	3.1	1.9
Terpinen-4-ol (11)	*(S)*1248/*(R)*1253	2.2	1.6	1.0	NS	NS	2.4	2.1	2.0	1.2	2.6	2.3	2.0	1.3
Lavandulol (12)	*(S)*1250/*(R)*1273	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E
α–Terpineol (13)	*(S)*1296/*(R)*1309	6.0	3.2	2.8	2.5	1.9	6.9	5.6	5.5	4.2	6.5	5.6	4.7	4.0
Lavandulyl acetate (14)	*(R)*1259/*(S)*1263	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E	1E

NS: not baseline separated.
1E: only (R) enantiomer found.
[a]: obtained on the reference column.

Table 4 retention time (t_R) and resolution (R_s) of the enantiomers of the lavender essential oil chiral markers under several single-ramp heating rates. Conditions: conventional column, FID. Legend: [1,2,3] = coeluting peaks; 1E = only one enantiomer detected; NR = not resolved.

Initial flow rate (mL/min)		**1**		**2**		**2**		**2**		**2**	
Heating rate (°C/min)		**2.0**		**2.0**		**2.6**		**3.3**		**5.0**	
Compound		**t_R (min)**	**R_s**	**t_R (min)**	**R_s**	**t_R (min)**	**R_s**	**t_R (min)**	**R_s**	**t_R (min)**	**R_s**
1a	(*S*)-α-Pinene	13.14	1.0	9.84	1.0	8.70	1.0	7.85	0.9	6.45	0.6
1b	(*R*)-α-Pinene	13.02		9.74		8.62		7.78		6.41[1]	
2a	(*S*)-Camphene	12.90	7.5	9.52	6.9	8.49	6.2	7.70	6.8	6.41[1]	5.0
2b	(*R*)-Camphene	13.65		10.21		9.04		8.15		6.71	
3a	(*S*)-β-Pinene	14.93	4.5	11.33	4.9	9.96	4.4	8.92	4.0	7.27	3.3
3b	(*R*)-β-Pinene	14.40		10.82		9.56		8.60		7.06	
4a	(*S*)-β-Phellandrene	20.85	5.1	16.88	5.9	14.38	5.5	12.49	4.9	9.64[2]	2.7
4b	(*R*)-β-Phellandrene	20.22		16.20		13.87		12.11		9.48	
5a	(*S*)-Limonene	20.65	6.5	16.65	6.8	14.21	6.7	12.37	6.5	9.64[2]	6.0
5b	(*R*)-Limonene	21.55		17.48		14.91		12.92		9.98	
6	1-Octen-3-ol	24.43	NR	20.67	NR	17.22	NR	14.67	NR	11.04	NR
7b	(*R*)-Camphor	25.86	1E	21.40	1E	18.02	1E	15.51	1E	11.88	1E
8a	(*S*)-Linalool	28.23	6.3	24.26[1]	7.0	20.05	6.1	16.96	5.5	12.63	4.1
8b	(*R*)-Linalool	27.28		23.27		19.28		16.37		12.23	
9a	(*S*)-Borneol	28.86	2.9	24.26[1]	4.5	20.30	3.5	17.33	2.7	13.10	2.3
9b	(*R*)-Borneol	29.30		24.80		20.66		17.59		13.25	
10a	(*S*)-Linalyl acetate	31.54	2.0	26.92	3.0	22.34	2.6	18.99	2.0	14.07[3]	NR
10b	(*R*)-Linalyl acetate	31.20		26.53		22.06		18.74		14.07[3]	
11a	(*S*)-Terpinen-4-ol	31.84	2.0	27.50	2.2	22.68	2.2	19.14	1.9	14.21	1.6
11b	(*R*)-Terpinen-4-ol	32.12		27.80		22.91		19.31		14.32	
12b	(*R*)-Lavandulyl acetate	33.02[1]	1E	28.17	1E	23.37	1E	19.83	1E	14.84	1E
13b	(*R*)-Lavandulol	33.02[1]	1E	29.02	1E	23.74	1E	19.87	1E	14.59	1E
14a	(*S*)-α-Terpineol	34.53	5.0	30.24	6.1	24.78	5.5	20.77	4.9	15.27	3.9
14b	(*R*)-α-Terpineol	35.19		30.96		25.30		21.17		15.52	

Table 5: method parameters (initial flow rates and translated heating rates) and measured parameters (analysis times and resolutions of α-pinene enantiomers).

Column dimensions (detector)	**25 m × 0.25 mm (FID)**											**10 m × 0.1 mm**	
												(FID)	**(MS)**
Initial flow rate (mL/min)	**2.0**	**0.3**	**0.5**	**0.7**	**1.0 (EOF)**	**1.4 (SOF)**	**1.7**	**2.3**	**2.5**	**2.8**	**4.0**	**0.56 (SOF)**	**0.56 (SOF)**
Temperature program													
Initial Temperature (°C)	50	50	50	50	50	50	50	50	50	50	50	50	50
Heating Rate1 (°C/min)	2.60	0.58	0.90	1.19	1.57	2.02	2.32	2.86	3.02	3.25	4.08	5.53	5.90
Intermediate T_1 (°C)	74	74	74	74	74	74	74	74	74	74	74	74	74
Heating Rate2 (°C/min)	3.30	0.74	1.14	1.51	2.00	2.57	2.95	3.63	3.83	4.13	5.17	7.04	7.50
Intermediate T_2 (°C)	115	115	115	115	115	115	115	115	115	115	115	115	115
Heating Rate3 (°C/min)	15.00	3.34	5.20	6.87	9.08	11.67	13.40	16.49	17.43	18.77	23.52	31.96	34.10
Final Temperature (°C)	220	220	220	220	220	220	220	220	220	220	220	220	220
Final Time (min)	2.00	8.98	5.77	4.37	3.30	2.57	2.24	1.82	1.72	1.60	1.27	0.94	0.90
Analysis time (min)	22.76	102.13	65.93	49.82	37.65	29.28	25.49	20.69	19.59	18.19	14.50	10.78	10.09
Resolution of α-pinene	0.96	1.01	1.04	1.06	1.07	1.04	1.00	0.93	0.90	0.88	0.72	1.09	1.10

Table 6: retention time (t_R), resolution (R_s) and *s* values of the enantiomers of the lavender essential oil chiral markers analysed under different conditions. Legends: 1E = only one enantiomer detected; NR = not resolved.

Column, initial flow rate		25m, 1 mL/min (EOF)			25m, 1.4 mL/min (SOF)			10m, 0.56 mL/min (SOF)					
								FID			MS		
Compound		t_R	σ	R_s	t_R	σ	R_s	t_R	σ	R_s	t_R	σ	R_s
1a	**(*S*)-α-Pinene**	14.43	1.91	1.1	11.21	1.54	1.0	4.07	0.48	1.1	3.81	0.47	1.0
1b	**(*R*)-α-Pinene**	14.29	1.95		11.10	1.56		4.04	0.51		3.78	0.47	
2a	**(*S*)-Camphene**	14.07	1.96	6.8	10.93	1.64	6.5	3.97	0.53	7.5	3.71	0.55	6.9
2b	**(*R*)-Camphene**	14.98	2.04		11.64	1.63		4.23	0.53		3.97	0.55	
3a	**(*S*)-β-Pinene**	16.50	2.11	4.7	12.82	1.67	4.7	4.66	0.54	5.4	4.37	0.48	5.4
3b	**(*R*)-β-Pinene**	15.84	2.05		12.31	1.60		4.47	0.51		4.19	0.50	
4a	**(*S*)-β-Phellandrene**	23.24	2.00	5.5	18.06	1.55	5.5	6.61	0.53	6.0	6.19	0.41	6.2
4b	**(*R*)-β-Phellandrene**	22.51	2.00		17.49	1.55		6.40	0.55		6.00	0.52	
5a	**(*S*)-Limonene**	23.01	1.99	7.3	17.88	1.55	7.3	6.54	0.52	7.8	6.13	0.47	8.4
5b	**(*R*)-Limonene**	24.01	2.17		18.66	1.63		6.84	0.60		6.40	0.51	
6	**1-Octen-3-ol**	27.21	1.87	NR	21.16	1.47	NR	7.79	0.53	NR	7.29	0.46	NR
7b	**(*R*)-Camphor**	28.53	2.26	1E	22.18	1.71	1E	8.13	0.67	1E	7.62	0.59	1E
8a	**(*S*)-Linalool**	31.14	1.90	6.0	24.21	1.49	6.0	8.93	0.51	5.6	8.36	0.47	5.8
8b	**(*R*)-Linalool**	30.09	3.32		23.41	2.45		8.61	1.17		8.07	1.00	
9a	**(*S*)-Borneol**	31.67	2.47	3.1	24.62	1.82	3.1	9.05	0.73	3.1	8.48	0.82	2.6
9b	**(*R*)-Borneol**	32.14	2.26		24.99	1.75		9.19	0.64		8.60	0.60	
10a	**(*S*)-Linalyl acetate**	34.46	1.64	3.0	26.79	1.30	3.0	9.84	0.48	3.1	9.20	0.51	2.7
10b	**(*R*)-Linalyl acetate**	34.09	2.17		26.50	1.65		9.73	0.61		9.11	0.55	
11a	**(*S*)-Terpinen-4-ol**	34.80	2.12	2.1	27.06	1.65	2.0	9.98	0.60	2.0	9.34	0.49	2.2
11b	**(*R*)-Terpinen-4-ol**	35.09	2.03		27.28	1.72		10.06	0.56		9.41	0.52	
12b	**(*R*)-Lavandulyl acetate**	35.90	2.06	1E	27.91	1.65	1E	10.25	0.53	1E	9.59	0.56	1E
13b	**(*R*)-Lavandulol**	36.06	1.82	1E	28.04	1.47	1E	10.33	0.44	1E	9.67	0.43	1E
14a	**(*S*)-α-Terpineol**	37.22	1.45	4.6	28.94	1.15	4.5	10.66	0.36	5.6	9.97	0.35	5.3
14b	**(*R*)-α-Terpineol**	37.65	1.36		29.28	1.08		10.78	0.32		10.09	0.31	
Notes: All t_R values are in minutes, all σ values are in seconds.													