Cyclodextrin Derivatives as Stationary Phases for the GC Separation of Enantiomers in the Flavor and Fragrance Field

Cyclodextrin derivatives as stationary phases for enantiomer GC separation of volatiles in the flavour and fragrance field

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This chapter will examine the chiral recognition of volatile odorants in the flavour and fragrance field, by enantioselective gas chromatography (Es-GC) with cyclodextrin derivatives as chiral stationary phases. The first part looks at enantiomers and odour, the need for chiral recognition, and the evolution of chiral stationary phases for Es-GC. This is followed by a more detailed discussion of cyclodextrins and the theoretical aspects of enantiomer separation, and applications of these techniques to Es-GC. The next part deals with the strategy of routine chiral recognition using cyclodextrin derivatives as chiral stationary phases; this is also illustrated through some examples concerning real-world complex samples. This part briefly describes i) the potential of multidimensional techniques in routine analysis, ii) enantiomer automatic identification and recognition, iii) the role played by mass spectrometry in Es-GC-MS, iv) fast Es-GC in chiral recognition, and v) the use of total analysis systems in chiral recognition.

1. Introduction

Metabolic processes in plants or animals are almost always stereospecific, and the resulting metabolites are very often chiral and present an enantiomeric excess. Enantiomer recognition, and the determination of enantiomeric excess (ee) and/or ratio (er) of a chiral compound, are therefore important steps to characterize a matrix and its biological activity, in particular in the fields of food, flavour and fragrance. Enantiomeric recognition is important: (i) to correlate chemical composition to organoleptic properties; (ii) to define the biosynthetic pathway of a metabolite, (iii) to characterize a matrix; (iv) to determine the geographic origin of a “natural” sample, and (v) to implement quality control and detect frauds or adulteration of “natural” samples[1].

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

Gas chromatography (GC), in particular when combined with mass spectrometry (MS), is currently the preferred technique to study the composition of the volatile fraction of natural product samples. However, enantiomer separation with GC requires chiral stationary phases (CSPs) able to interact in different ways with each enantiomer of the investigated chiral compound(s). Several chiral selectors operating on different principles have been proposed for Es-GC, and three selectors, distinguishable for their selector-selectand interaction mode, have been successfully applied to routine separation of enantiomers, i.e.[2]:

- chiral amino acids via hydrogen bonding;
- chiral metal coordination compounds via complexation;
- cyclodextrin derivatives via inclusion (inter alia).

Cyclodextrin derivatives (CDs) are the most popular chiral stationary phases (CSPs) in the flavour and fragrance field today, because of their wide range of applicability and high enantioselectivity. These characteristics make it possible to separate the enantiomers of a large number of chiral molecules with different structures and organic functions, without the need for preliminary derivatization to the corresponding diastereoisomers. The variety of CSPs now available means that most enantiomer pairs can be separated.

3. Chiral stationary phases based on (inter alia) inclusion - Cyclodextrin derivatives as chiral selectors for Es-GC

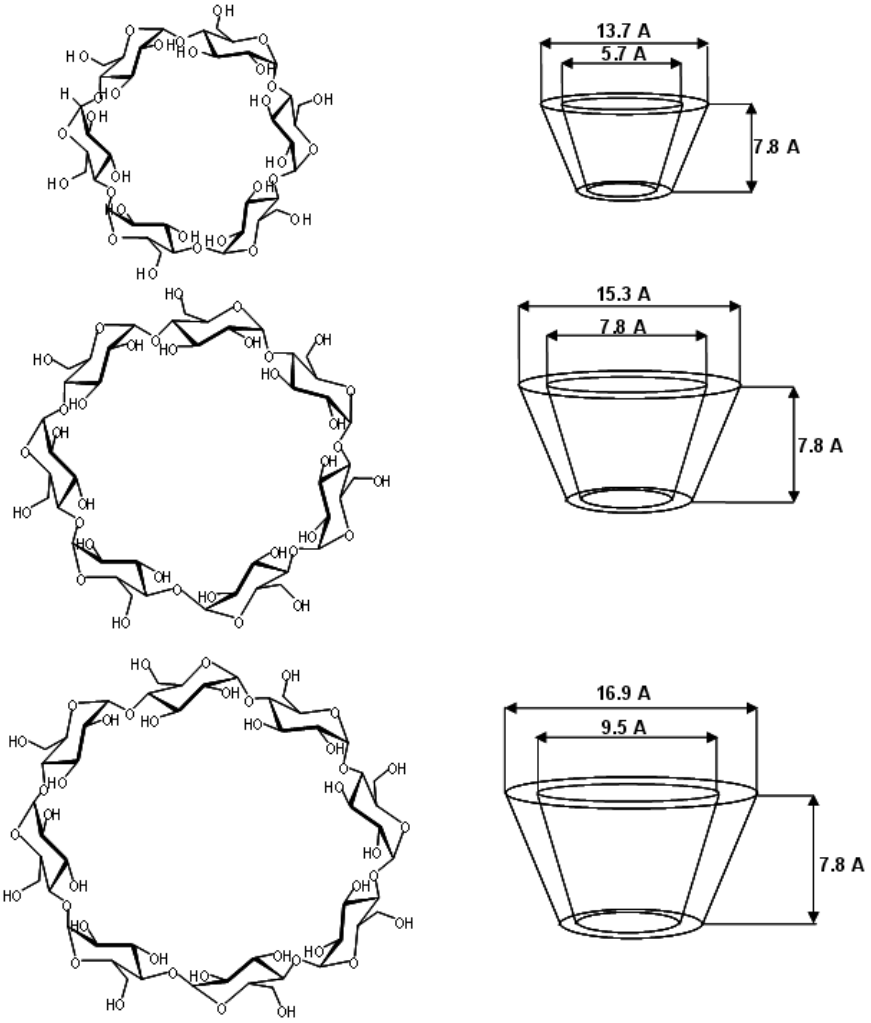

Figure 1 - Structure of α-, β- and γ-cyclodextrines

Cyclodextrins (CDs), also known as cycloamyloses, cycloglucanes, or cyclomaltoligoses, are a homologous series of non-reducing cyclic oligosaccharides made up of six to twelve (β)-D-glucopyranose units linked by an α-1-4-glycoside bond, deriving from the enzymatic degradation of the starch polysaccharide by cyclodextrin glycosyltransferase, from either *Klebsiella pneumonia* or *Bacillus macerans*. They consist of 6-12 sugar units; the best known and most widely available CDs are those with six (α-CD), seven (β-CD) or eight (γ-CD) sugar units (Figure 1).

The first chiral separation in GC using CD as stationary phases was due to Koscielski and Sybilska[3]; 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 next applied to capillary gas chromatography, almost contemporarily by Juvancz's and Schurig's groups in 1987[4-5] and 1988[6]. Two different approaches were adopted to provide columns with high efficiency and enantioselectivity:

(a) use of CD derivatives that are liquid at room temperature, e.g., per-*n*-pentylated cyclodextrins[7-8].

(b) use of solid or semisolid persubstituted-β or γ-cyclodextrins diluted in moderately polar polysiloxanes (e.g., OV-1701)[6, 9-12] in order to combine the high enantioselectivity of the modified CDs with the very good gas chromatographic properties of polysiloxanes. Dilution in polysiloxane is almost the only approach now adopted for routine use.

3.1. Chemistry of cyclodextrin derivatives as chiral selector 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. Several derivatives have been described, mainly based on β-CDs, but a universally-applicable derivative has not yet been found. A fundamental improvement was the introduction of bulky substituents (e.g., the *tert*-butyldimethylsilyl (TBDMS) or *tert*-hexyldimethylsilyl- (THDMS) groups)[13-14] at the primary C6-hydroxyl groups, first proposed by Blum and Aichholz[13], but in the main developed by Mosandl's group[14]. 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, where the substituents at the C2- and C3-secondary hydroxyl groups drive the enantioselective interaction.

Several approaches to improve enantioselectivity and chromatographic results have been attempted: a) synthesis of "fully asymmetrically substituted derivatives"[15], i.e., with different substituents in the C2- and C3- secondary hydroxyl groups, and a bulky substituent (e.g., *t*-butyldimethylsilyl-, TBDMS) in the C6- primary hydroxyl groups, to obtain synergic enantioselectivity, b) mixing two CDs or condensing two CD units, although in this case the resulting enantioselectivity was not as satisfactory as expected[16-17]; c) mixing two structurally-different chiral selectors, e.g., amino acid derivatives and cyclodextrin derivatives[18-20], to extend the range of enantioselectivity, and d) cyclodextrin selector(s) chemically and permanently linked to a polysiloxane backbone[21-23] (Chirasil-Dex).

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

Chiral recognition with modified cyclodextrins is a multimodal process and may, among others, involve inclusion, hydrogen-bonding, dispersion forces, dipole–dipole interactions, electrostatic interactions, and hydrophobic interactions[24-25]. The separation of enantiomers is due to the differences in the low-energy interaction between the diastereomeric CD selector–selectand (enantiomer) association equilibria; the high efficiency of capillary GC columns also plays a fundamental role in achieving separation[26]. Important contributions to understanding the mechanisms of chiral recognition with CD derivatives were made by Schurig and coworkers, who developed a thermodynamic model to describe enantiomer separation[26-27], and by Lipkowitz et al.[28-29], who studied some aspects of the enantiomer separation with molecular modelling.

The thermodynamic results have a concrete impact on routine Es-GC, because the separation of enantiomers by ES-GC with CD derivatives as CSP is based on fast kinetics and is thermodynamically driven, implying that optimal enantiomer separation requires the lowest possible operation temperature.

4. Measurement of the enantiomeric distribution

The main aim of enantiomer recognition is to determine the precise distribution of the two enantiomers in the corresponding markers of a matrix. Enantiomeric distribution is usually expressed in terms of *enantiomeric excess* (*ee*), *enantiomeric composition* (*ec*) or *enantiomeric ratio* (*er*)[30]. *Enantiomeric excess*, also known as enantiomeric purity, expresses the super-abundance of one enantiomer over the other, and is defined as

$$ee = \frac{E_1 - E_2}{E_1 + E_2} \quad (1)$$

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

Enantiomeric composition (*ec*) is defined as the molar fraction of the major enantiomer in a mixture:

$$ec = x_{E_1} = \frac{E_1}{E_1 + E_2} \quad (2)$$

In this case too, ec is generally expressed as a percentage

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

$$er = \frac{E_1}{E_2} \quad (3)$$

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

$$ee = \frac{(er - 1)}{(er + 1)} \quad (4)$$

and

$$er = \frac{(1 + ee)}{(1 - ee)} \quad (5)$$

In routine analysis, correct measurement of the above parameters requires that the peaks of the two enantiomers be baseline separated, i.e., their resolution must be $R_S \geq 1.5$.

5. Enantioselective GC analysis with cyclodextrins in the flavour and fragrance field

Cyclodextrin derivatives are the most widely used chiral selectors in Es-GC in the flavour and fragrance field. Their advantages and disadvantages are well known; their main advantages being:

- they can separate underivatized enantiomers, therefore real-world natural product samples can be analysed directly without further 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;
- the CD-based CSP columns have good chromatographic performance (efficiency and inertness) and operate at a wide range of temperatures, thanks to their dilution in moderately-polar polysiloxanes.

However, CDs also have some disadvantages:

- the absence of a "universal" cyclodextrin derivative separating most chiral compounds; a laboratory must therefore have a number of columns coated with different CD-based CSPs;
- difficulty of identifying enantiomers and measuring ee and/or er in real-world complex samples with mono-dimensional Es-GC, because of the increased possibility of peak overlapping, exacerbated by the double number of peaks when chiral analyte(s) are separated;
- long analysis time, due to the need for long columns and low temperature program rates, because of the cyclodextrin mechanism of separation.

Partly in view of these disadvantages, the next three paragraphs will examine the optimization of strategies for the chiral recognition of volatile markers by Es-GC and Es-GC-MS, with CD as chiral selectors, in complex mixtures in the flavour and fragrance field; the observations are mainly based on the authors' everyday experience.

6. Analysis of enantiomers in complex samples

Two complementary but distinct approaches are available for the qualitative (and, as a consequence, in some cases quantitative) recognition of enantiomers in complex samples: i) the first is to adopt a second dimension in separation (by conventional heart-cut GC-GC or comprehensive 2D GC), ii) the second approach is to use a second dimension in detection, by coupling GC with mass spectrometry (MS)[31]. The latter method applies a strategy that is the converse of the conventional one, i.e., the enantiomer(s) are located in the chromatogram by their MS spectra and identified by GC data (i.e Kovats retention indices (*Is*)[32] or linear retention indices ($I^T s$))[33]. This is because, as is known, MS is not a chiral probe, making the mass spectra of two enantiomers indistinguishable. Conversely, chromatographic data (and in particular linear retention indices) are reliable and effective parameters for enantiomer identification, being characteristic for each analyte (enantiomer); similar considerations can be made with locked retention times[34] (see below).

From these considerations, an MS library specific for the identification of enantiomer components in the flavour and fragrance field has been developed at an interlaboratory level, using $I^T s$ values "interactively" in parallel to MS spectra[31]. It is based on the interactive I^T/mass spectrum system[35] developed by Costa et al.[36] for the flavour and fragrance field: $I^T s$ are calculated automatically 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 (determined preliminarily) within which its I^T must fall (Retention Index Allowance (RIA)). Four cyclodextrin derivatives diluted at 30% in PS-086 were used to separate a large number of racemates usually analysed in the flavour and fragrance field; these are:

i) $6^{I\text{-}VII}$-*O*-methyl-$3^{I\text{-}VII}$-*O*-pentyl-$2^{I\text{-}VII}$-*O*-methyl-β-cyclodextrin[37-38],

ii) $6^{I\text{-}VII}$-*O*-TBDMS-$3^{I\text{-}VII}$-*O*-methyl-$2^{I\text{-}VII}$-*O*-methyl-β-cyclodextrin[39],

iii) $6^{I\text{-}VII}$-*O*-TBDMS-$3^{I\text{-}VII}$-*O*-ethyl-$2^{I\text{-}VII}$-*O*-ethyl-β-cyclodextrin[40],

iv) $6^{I\text{-}VII}$-*O*-TBDMS-$3^{I\text{-}VII}$-*O*-acetyl-$2^{I\text{-}VII}$-*O*-acetyl-β-cyclodextrin[39].

The library consists of 134 racemates whose I^T values were determined on four columns coated with these four CD chiral selectors. Table 1 reports the list of racemates included in the first version of the library.

The same approach can be adopted with another software package, AMDIS, (Automatic Mass Spectral Deconvolution), developed by the National Institute of Standards and Technology (USA)[41].

Retention time locking (RTL)[34] is an approach that provides reliable identification of an analyte from its GC retention data in programmed temperature analysis, based on the adjustment of the inlet pressure necessary to achieve the desired match in retention time(s) of an analyte(s) with similarly configured GC systems.

Table 1: list of compounds included in the library[31]

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

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

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-phenylglycidate
Isobornyl acetate
Isobornyl isobutyrate
Lavandulyl acetate
Linalyl acetate
Linalyl cinnamate
Menthyl acetate
Methyl 3-hydroxyhexanoate
Methyl dihydrofarnesoate
Neomenthyl acetate
Nopyl acetate
Propylene glycolbutyrate

Aldehydes
Citronellal
Cyclamen aldehyde
Hydroxycitronellal
Myrtenal
Perillyl aldehyde

Heterocyles
Ambroxide
Menthofuran
Rose oxide

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

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

7. Fast Es-GC analysis with cyclodextrins as chiral stationary phases

As already mentioned, 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 program rates have to be applied.

Routine application requires the development of fast Es-GC methods, in order to run 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[42] (d_c: 100 μm). NB columns not only increase analysis speed and analyte detectability, because of peak sharpening[43], but also reduce the enantiomer elution temperature, because of their shorter length. Together, these result in a gain of enantioselectivity that compensates (in full or in part) for the loss of efficiency (N) due to column shortening. Fast enantiomer separations (even in a few seconds) were already achieved in the early 1990s, with short columns on low-complexity samples and/or those containing a limited number of enantiomers to be simultaneously analyzed[44-46]. With medium-to-high complexity samples, as 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 measure ee and/or er correctly. This paragraph discusses the two complementary methods developed and adopted in the authors' laboratory to speed up routine Es-GC analyses[47-48]. The two methods are illustrated through the analysis of rosemary and bergamot essential oils (e.o.s).

The first approach[48] consists of searching for the best trade-off between speed of analysis and loss of resolution of chiral compounds. This may be at the expense of the separation of the other sample components, provided that baseline separation of the enantiomers of the chiral compound(s) investigated is maintained, in order to determine *ee* or *er* correctly. MS therefore plays a crucial role, because it can highlight the separated enantiomers by operating in extract-ion, SIM- or MIM-MS modes. Analysis time is reduced by exploiting the excess of resolution obtained with columns coated with the latest-generation CD derivatives ($6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-*O*-ethyl-$3^{I\text{-}VII}$-*O*-methyl-β-CD in PS086) simultaneously applied to the chiral recognition of several chiral compounds ("the one column for one problem" approach)[49], by acting on column dimension, flow rate and temperature program.

Rosemary essential oil is characterized by several chemotypes that contain several chiral components[50]; the sample investigated contained, among others, camphor, linalool, borneol, terpinen-4-ol, and α-terpineol.

Figure 2 shows the part of the Es-GC–MS profiles of the rosemary essential oil where chiral markers elute, together with the identification of peaks of chiral components.

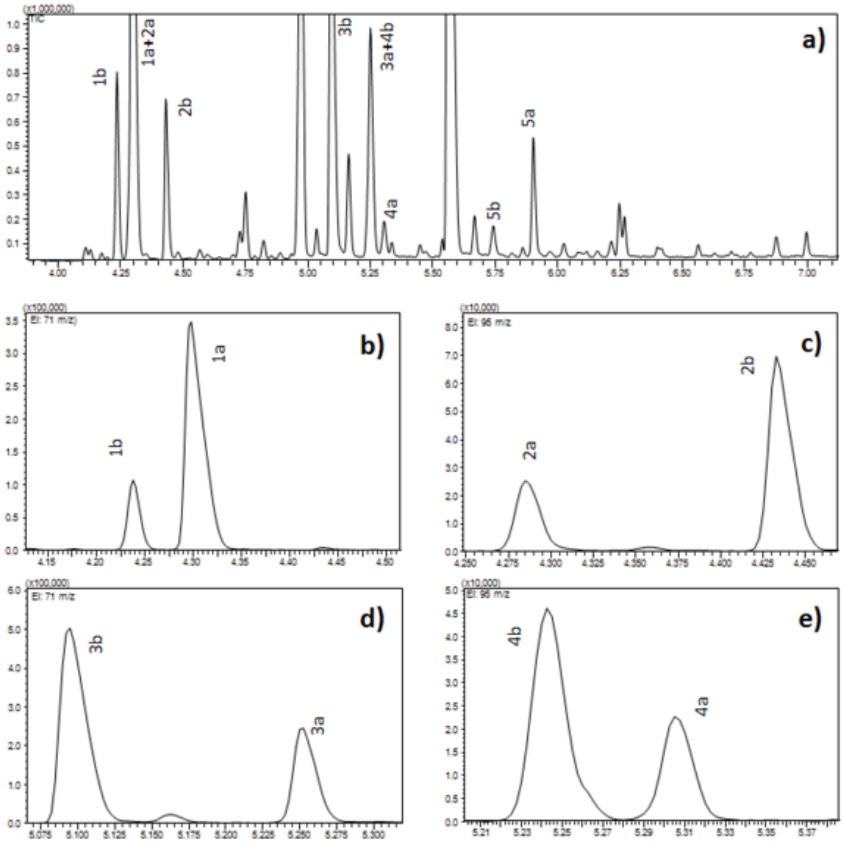

Figure 2- (a) Es-GC–MS profile of the rosmary e.o. Column: 30% 6$^{I\text{-}VII}$-O-TBDMS-2$^{I\text{-}VII}$-O-ethyl-3$^{I\text{-}VII}$-O-methyl-β-CD in PS086 (10m×0.10mm d_c, 0.10 μm d_f); flow rate: 1 mL/min; temperature program: 50°C-10°C/min-220°C); extract ion profiles of (b) linalool (71m/z), (c) camphor (95m/z), (d) terpinen-4-ol (71 m/z), (e) borneol (95m/z). Peak identification: 1: camphor 2: linalool, 3: borneol, 4: terpinen-4-ol, 5: α terpineol; a: (R) enantiomer, b: (S) enantiomer.

The results of the analysis on a NB column (l: 10m, d_c: 0.10mm, d_f: 0.10 µm) at 10°C/min show that the analysis time is reduced to 7 minutes, but that (*R*)-camphor (1a) and (*R*)-linalool (2a) and (*R*)-borneol (3a) and (*S*)-terpinen-4-ol (4b) coelute; conversely, though, the selected extract ion profiles are base-line separated, thus affording a correct *er* or *ee* determination.

The second approach[47] is based on the opposite strategy, i.e., the analysis time is shortened by seeking to maximize separation efficiency of the chromatographic system, by optimizing analysis conditions. Routine analyses of large numbers of different samples in a given 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, with a dedicated method for each matrix, can successfully speed up routine GC analyses considerably. This approach is based on the theoretical and practical studies done by Blumberg and co-workers on fast-GC[51-52], which resulted in the well-known GC method-translation[53]. This method requires the optimal temperature program to be defined for a given sample; from this is determined the flow-rate producing the highest efficiency (i.e., the plate number) of a given column (efficiency-optimized flow, EOF), or flow-rate, column dimensions, and carrier gas corresponding to the shortest analysis time for a given required plate number (speed-optimized flow, SOF[51, 53]).

The optimization of Es-GC analysis conditions of bergamot essential oil with a conventional column (30% $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-*O*-ethyl-$3^{I\text{-}VII}$-*O*-methyl-β-CD in PS086, l: 25m, d_c: 0.25mm, d_f: 0.25 µm) involved 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.

Bergamot essential oil is characterized by seven main chiral markers α-pinene (1), β-pinene (2), sabinene (3), limonene (4), linalool (5), linalyl acetate (6), and α-terpineol (7)[54]. Under the initial analysis conditions, i.e., with the conventional column and using routine reference conditions (flow rate: 1 mL/min, temperature program: 50°C-220°C at 2°C/min), bergamot essential oil presented two non-separated critical clusters: (*R*)-β-pinene (2a), (*R*)-sabinene (3a), and β-myrcene, and (*R*)-α-terpineol (7a) and neral. Figure 3 reports the Es-GC patterns of the bergamot essential oil analysed (a) under routine conditions and (b) under optimized multi-rate temperature program with the conventional column and (c) under optimized conditions after method translation with a NB column (l: 10m, d_c: 0.10mm, d_f: 0.10 µm). The optimized temperature program with the conventional column (50°C//2.33°C/min //102°C//10.4°C/min//220°C) provided the separation of (*R*)-α-terpineol (7a) from neral but not that of (*R*)-β-pinene (2a) from β-myrcene. 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)[55]. SOF can be calculated from EOF with the equation SOF = $\sqrt{2}$ EOF[55], i.e., the initial EOF was here 1 mL/min, so that the initial SOF was 1.4 mL/min, and the corresponding heating rates were 2.76 and 12.55°C/min. Under these conditions, the analysis time was about 23 min.

The optimized EOF method with a conventional ((l: 25m, d_c: 0.25mm, d_f: 0.10µm) column was then translated to the corresponding NB column (l: 10m, d_c: 0.10mm, d_f: 0.10 µm). The flow rate was reduced in proportion to the column d_c, i.e., from 1.0 mL/min to 0.40 mL/min, thus assuring EOF operation of the NB column. Under these conditions, analysis time was reduced to something more than a third (i.e., retention time of the last investigated peak was reduced from about 35 min to 13 min), with an improvement of peak resolution and separation of (*R*)-β pinene (2a) from β-myrcene.

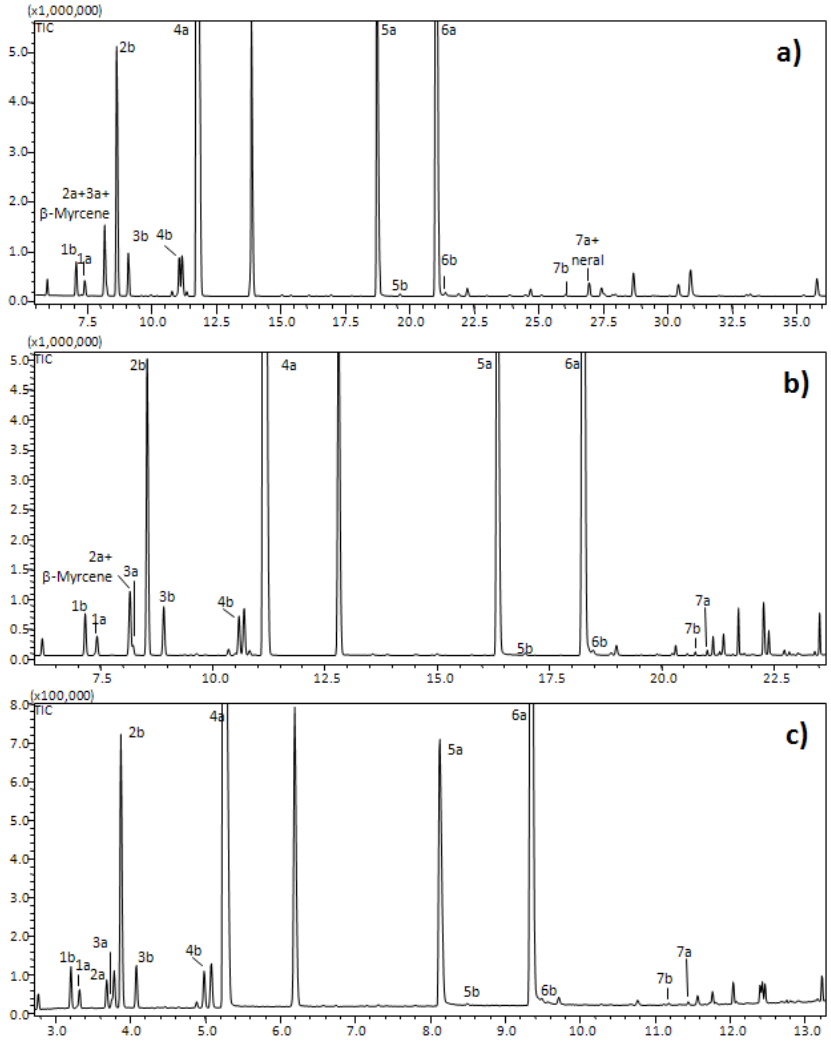

Figure 3- Es-GC–MS profile of a bergamot e.o.; column: 30% 6$^{I\text{-}VII}$-O-TBDMS-2$^{I\text{-}VII}$-O-ethyl-3$^{I\text{-}VII}$-O-methyl-β-CD in PS086 (25m×0.25mm d_c, 0.25 μm d_f); a) reference conditions (flow: 1 mL/min, temp. prog.: 50°C-2°C/min-220°C), b) optimized cond. conventional d_c column (flow: 1 mL/min, temp. prog.: 50°C-2.33°C/min-102-10.4°/min-220°C), c) optimized cond. 10m NB column (flow: 0.4 mL/min, temp. prog.: 50°C-4.74°C/min-102-21.12°C/min-220°C). Peak identification: 1: α-pinene 2: β-pinene, 3: sabinene, 4: limonene, 5: linalool, 6: linalyl acetate, 7: α-terpineol; a: (R) enantiomer, b: (S) enantiomer.

8. Bornyl acetate, a common chiral compound whose enantiomers are difficult to separate

The last example aims to emphasize the role of the diluting phase in chiral recognition with CDs as chiral selector. It concerns the "intriguing" story of the chiral recognition of camphor, borneol, isoborneol, iso-bornyl acetate, and bornyl acetate (Figure 4). While the first four are currently separated at least by one of the four CSPs used to build up the above automatic database, bornyl acetate is one of the few compounds that were not separated at all by one of them (see § 6), thus making it necessary to explore other CSPs available in the library of columns of the authors' laboratory.

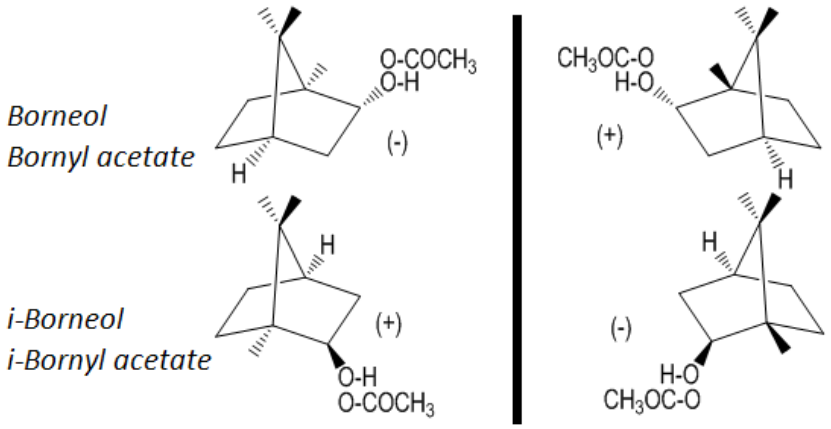

Figure 4- Absolute configuration of borneol, i-borneol, bornyl acetate and i-bornylacetate

Several attempts were made, and separation was achieved only with a conventional 50% $6^{I\text{-}VIII}$-*O*-THDMS-$3^{I\text{-}VIII}$-*O*-acetyl-$2^{I\text{-}VIII}$-*O*-acetyl-γ-CDX but diluted in OV-1701 (Figure 5c) column (l: 40 m, d_c: 0.20 mm, d_f: 0.20µm) in 93 minutes; Figure 5b shows that the same CD but diluted in PS-086 was not successful at all. The adoption of the strategy of optimization with a narrower and shorter column (column: l: 20m, d_c: 0.15 mm, d_f: 0.15 µm) enabled the analysis time to be reduced to 35 minutes, while keeping the base-line separation of all five chiral compounds (Figure 5d). This example is a clear demonstration that the true enantioselective stationary phase is the system CD/diluting phase, although the chiral discrimination is of course due to the CD chiral selector.

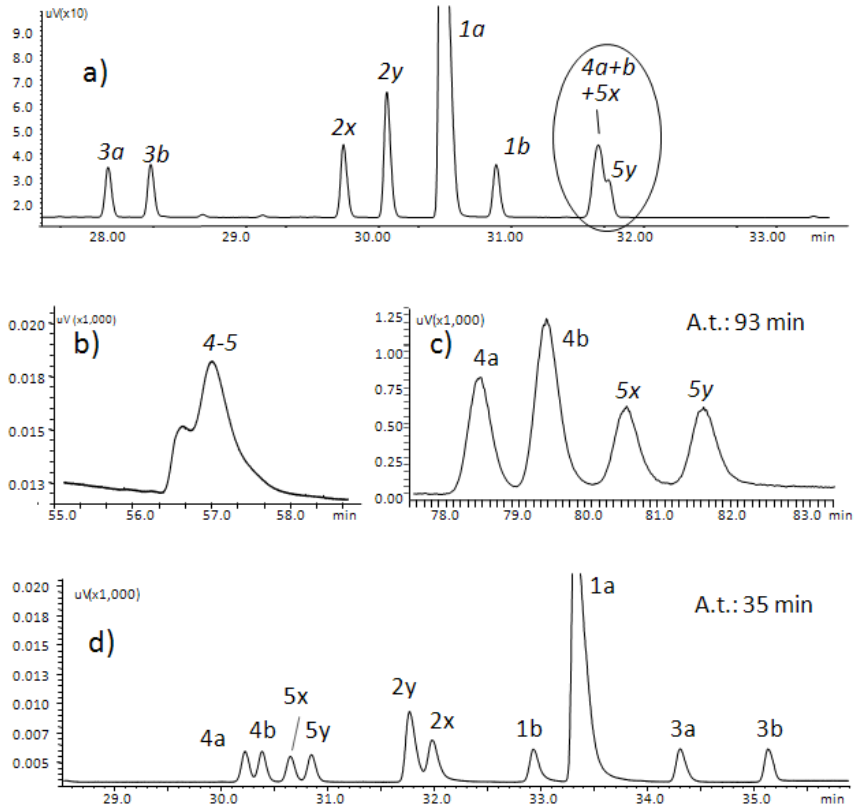

Figure 5: Analyte identification: 1: borneol, 2: i-borneol, 3: camphor, 4: bornyl acetate; 5: i-bornyl acetate, a: (-); b: (+); x and y: configuration not attributed; A.T. analysis time. a) Column: ***30% 6$^{I\text{-}VII}$-O-TBDMS-3$^{I\text{-}VII}$-O-ethyl-2$^{I\text{-}VII}$-O-ethyl-β-CDX in PS086****; l: 25m, d_c: 0.25 mm, d_f: 0.25 µm, Anal. cond.: temp. prog.: 50°C/2°C/min/200°C , carrier: H_2; flow rate: 1.0 mL/min; b)Column:* ***50%6$^{I\text{-}VIII}$-O-THDMS-3$^{I\text{-}VIII}$-O-acetyl-2$^{I\text{-}VIII}$-O-acetyl-γ-CDX in PS086****; l: 25m, d_c: 0.25 mm, d_f: 0.25 µm; Anal. cond.: temp. prog.: 80°C/2°C/min/200°C; carrier: H_2; flow rate: 1.0 mL/min; c) Column:* ***50% 6$^{I\text{-}VIII}$-O-THDMS-3$^{I\text{-}VIII}$-O-acetyl-2$^{I\text{-}VIII}$-O-acetyl-γ-CDX in OV-1701; l: 40m, d_c: 0.20 mm, d_f: 0.20 µm****; Anal. cond.: temp. prog.: 70°C/0.4°C/min/200°C, carrier: H_2; flow rate: 0.8 mL/min; d) Column:* ***50% 6$^{I\text{-}VIII}$-O-THDMS-3$^{I\text{-}VIII}$-O-acetyl-2$^{I\text{-}VIII}$-O-acetyl-γ-CDX in OV-1701****; l: 20m, d_c: 0.15 mm, d_f: 0.15 µm****, Anal. cond.: temp. progr.: 70°C/1°C/min/200°C; carrier: H_2; flow rate: 0.6 mL/min*

9. Total Analysis Systems and real-world sample analysis

The above paragraphs show that Es-GC can be very effective as a technique for chiral recognition in the flavour and fragrance field, and that it can be adapted to modern strategies of analysis based on fully-automatic systems, better known as "Total Analysis Systems" (TAS). TAS are systems in which the three main steps of the analytical process (sample preparation, analysis, and data processing) are integrated on-line into a single step[56-57].

Adoption of these systems has been made possible by the parallel improvements achieved not only in Es-GC but also in sample preparation and mass spectrometry as detector. In particular, the volatile nature of most odorants in the flavour and fragrance field makes headspace sampling, when applicable, the technique of choice for these analyses[58-60]. HS techniques have the advantages of being solvent-free, fast, simple, reliable, and, above all, easy to automate and to combine on-line to GC-MS systems. These techniques include both conventional (static (S-HS) or dynamic (D-HS)

modes, and High Concentration Capacity Headspace (HCC-HS) techniques[58, 60]; the latter act as a "bridge" between S-HS and D-HS, in which volatiles are statically or dynamically accumulated on polymers, such as polydimethylsiloxane (PDMS), operating in sorption and/or adsorption modes or, less frequently, they are accumulated on solvents (HS-SPME, HSSE, HS-STE, HS-LPME, etc.)[61]. A successful example of this strategy is the authentication of fruit juices and flavoured foods through the γ-lactone fraction by fast Es-GC-MS analysis combined on-line with HS-SPME which involves all the concepts mentioned above[62].

10. Conclusions

Cyclodextrin derivatives are nowadays the most effective chiral stationary phases available for Es-GC in the flavour and fragrance field, because of their i) high stability and separation repeatability, ii) acceptable analysis times, iii) high inertness with several classes of compounds of different polarities, and iv) extended range of operative temperatures (20-250°C). However, much work remains to be done, mainly to further increase our understanding of their mechanisms of enantiomer recognition[63], and to design a new generation of CD derivatives with a more universal enantioselectivity, so as to extend, for instance, their use to highly polar chiral compounds.

References

1. Koppenhoefer, B.; Behnisch, R.; Epperlein, U.; Holzschuh, H.; Bernreuther, A.; Piras, P.; Roussel, C. Enantiomeric odor differences and gas chromatographic properties of flavors and fragrances. *Perfum. Flavor.* **1994,** *19*, 1-14.

2. Schurig, V. Separation of enantiomers by gas chromatography. *J. Chromatogr. A* **2001,** *906*, 275-299.

3. Koscielski, T.; Sybilska, D.; Jurczak, J. Separation of α-and β-pinene into enantiomers in gas-liquid chromatography systems via α-cyclodextrin inclusion complexes. *J. Chromatogr.* **1983,** *280*, 131-134.

4. Alexander, G.; Juvancz, Z.; Szejtli, J. Cyclodextrins and their derivatives as stationary phases in GC capillary columns. *HRC & CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* **1988,** *11*, 110-113.

5. Juvancz, Z.; Alexander, G.; Szejtli, J. Permethylated β-Cyclodextrin as stationary phase in capillary gas chromatography. *J. High Res. Chromatog.* **1987,** *10*, 105-107.

6. Schurig, V.; Nowotny, H. P. Separation of enantiomers on diluted permethylated β-Cyclodextrin by high-resolution gas chromatography. *J. Chromatogr.* **1988,** *441*, 155-163.

7. König, W. A.; Lutz, S.; Mischnick-Lübbecke, P.; Brassat, B.; Wenz, G. Cyclodextrins as chiral stationary phases in capillary gas chromatography. Part 1. Pentylated α-cyclodextrin. *J. Chromatogr.* **1988,** *447*, 193-197.

8. König, W. A.; Lutz, S.; Wenz, G. Modified cyclodextrins – novel highly enantioselective stationary phases for gas chromatography. *Angew. Chem. Int. Ed. Edit.* **1988,** *27*, 979-980.

9. Bicchi, C.; Artuffo, G.; D'Amato, A.; Manzin, V.; Galli, A.; Galli, M. Cyclodextrin derivatives in the GC separation of racemic mixtures of volatile compounds. Part VI: the influence of the diluting phase on the enantioselectivity of 2,6-di-*O*-methyl-3-*O*-pentyl-β-cyclodextrin. *J. High Res. Chromatog.* **1993,** *16*, 209-214.

10. Bicchi, C.; D'Amato, A.; Manzin, V.; Galli, A.; Galli, M. Cyclodextrin derivatives in GC separation of racemic mixtures of volatiles. Part IX. The influence of the different polysiloxanes as diluting phase for 2,3-di-*O*-acetyl-6-*O*-t-butyldimethylsilyl-β-cyclodextrin on the separation of some racemates. *J. Microcolumn Sep.* **1995,** *7*, 327-336.

11. Schurig, V.; Bürkle, W. Extending the scope of enantiomer resolution by complexation gas chromatography. *J. Am. Chem. Soc.* **1982,** *104*, 7573-7580.

12. Schurig, V.; Jung, M.; Schmalzing, D.; Schleimer, M.; Duvekot, J.; Buyten, J. C.; Peene, J. A.; Mussche, P. CGC enantiomer separation on diluted cyclodextrin derivatives coated on fused-silica columns. *J. High Resolut. Chromatog.* **1990,** *13*, 470-474.

13. Blum, W.; Aichholz, R. Gas chromatographic enantiomer separation on tert-butyldimethylsilylated β-cyclodextrin diluted in PS-086 - a simple method to prepare enantioselective glass-capillary columns. *J. High Resolut. Chromatog.* **1990,** *13*, 515-518.

14. Dietrich, A.; Maas, B.; Karl, V.; Kreis, P.; Lehmann, D.; Weber, B.; Mosandl, A. Stereoisomeric flavor compounds. Part 55. Stereodifferentiation of some chiral volatiles on heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*butyldimethylsiyl)-β-cyclodextrin. *J. High Resolut. Chromatog.* **1992,** *15*, 176-179.

15. Bicchi, C.; Cagliero, C.; Liberto, E.; Sgorbini, B.; Martina, K.; Cravotto, G.; Rubiolo, P. New asymmetrical per-substituted cyclodextrins (2-*O*-methyl-3-*O*-ethyl- and 2-*O*-ethyl-3-*O*-methyl-6-*O*-*t*-butyldimethylsilyl-β-derviatives) as chiral selectors for enantioselective gas chromatography in the flavour and fragrance field. *J. Chromatogr. A* **2010,** *1217*, 1106-1113.

16. Bayer, M.; Mosandl, A. Improved gas chromatographic stereodifferentiation of chiral main constituents from different essential oils using a mixture of chiral stationary phases. *Flavour Frag. J.* **2004,** *19*, 515-517.

17. Nie, M. Y.; Zhou, L. M.; Wang, Q. H.; Zhu, D. Q. Gas chromatographic enantiomer separation on single and mixed cyclodextrin derivative chiral stationary phases. *Chromatographia* **2000,** *51*, 736-740.

18. Levkin, P. A.; Levkina, A.; Schurig, V. Combining the enantioselectivities of L-valine diamide and permethylated β-cyclodextrin in one gas chromatographic chiral stationary phase. *Anal. Chem.* **2006,** *78*, 5143-5148.

19. Stephany, O.; Dron, F.; Tisse, S.; Martinez, A.; Nuzillard, J.-M.; Peulon-Agasse, V.; Cardinaël, P.; Bouillon, J.-P. (L)- or (D)-Valine *tert*-butylamide grafted on permethylated β-cyclodextrin derivatives as new mixed binary chiral selectors: Versatile tools for capillary gas chromatographic enantioseparation. *J. Chromatogr. A* **2009,** *1216*, 4051-4062.

20. Stephany, O.; Tisse, S.; Coadou, G.; Bouillon, J. P.; Peulon-Agasse, V.; Cardinael, P. Influence of amino acid moiety accessibility on the chiral recognition of cyclodextrin-amino acid mixed selectors in enantioselective gas chromatography. *J. Chromatogr. A* **2012,** *1270*, 254-261.

21. Armstrong, D. W.; Tang, Y. B.; Ward, T.; Nichols, M. Derivatized cyclodextrins immobilized on fused-silica capillaries for enantiomeric separations via capillary electrophoresis, gas chromatography, or supercritical fluid chromatography. *Anal. Chem.* **1993,** *65*, 1114-1117.

22. Donnecke, J.; Paul, C.; König, W. A.; Svensson, L. A.; Gyllenhaal, O.; Vessman, J. Immobilization of heptakis (6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin for capillary gas chromatography and supercritical fluid chromatography and micro-liquid chromatography. *J. Microcolumn Sep.* **1996,** *8*, 495-505.

23. Schurig, V.; Juvancz, Z.; Nicholson, G. J.; Schmalzing, D. Separation of enantiomers on immobilized polysiloxane-anchored permethylated-β-cyclodextrin (Chirasil-Dex) by supercritical fluid chromatography. *J. High Resolut. Chromatog.* **1991,** *14*, 58-62.

24. Berthod, A.; Li, W. Y.; Armstrong, D. W. Multiple enantioselective retention mechanisms on derivatized cyclodextrin gas chromatographic chiral stationary phases. *Anal. Chem.* **1992,** *64*, 873-879.

25. Schurig, V.; Nowotny, H. P. Gas chromatographic separation of enantiomers on optically active metal-complex-free stationary phases. Part 2. Gas chromatographic separation of enantiomers on cyclodextrin derivatives. *Angew. Chem. Int. Ed. Edit.* **1990,** *29*, 939-957.

26. Jung, M.; Schmalzing, D.; Schurig, V. Theoretical approach to the gas chromatographic separation of enantiomers on dissolved cyclodextrin derivatives. *J. Chromatogr.* **1991,** *552*, 43-57.

27. Schurig, V. Enantiomer separation by gas chromatography on chiral stationary phases. *J. Chromatogr. A* **1994,** *666*, 111-129.

28. Lipkowitz, K. B.; Coner, R.; Peterson, M. A. Locating regions of maximum chiral discrimination: A computational study of enantioselection on a popular chiral stationary phase used in chromatography. *J. Am. Chem. Soc.* **1997,** *119*, 11269-11276.

29. Lipkowitz, K. B.; Coner, R.; Peterson, M. A.; Morreale, A.; Shackelford, J. The principle of maximum chiral discrimination: Chiral recognition in permethyl-β-cyclodextrin. *J. Org. Chem.* **1998,** *63*, 732-745.

30. Schurig, V. Terms for the quantitation of a mixture of stereoisomers. *Enantiomer* **1996,** *1*, 139-143.

31. Liberto, E.; Cagliero, C.; Sgorbini, B.; Bicchi, C.; Sciarrone, D.; Zellner, B. D.; Mondello, L.; Rubiolo, P. Enantiomer identification in the flavour and fragrance fields by "interactive" combination of linear retention indices from enantio selective gas chromatography and mass spectrometry. *J. Chromatogr. A* **2008,** *1195*, 117-126.

32. Kovats, E. Gas chromarographische charakteriserung organischer verbindungen. Retention indices aliphatischer halogenide, alkohole, aldehyde und ketone. *Helv. Chim. Acta* **1958,** *41*, 1915-1932.

33. Van den Dool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963,** *11*, 463-471.

34. Blumberg, L. M.; Klee, M. S. Method translation and retention time locking in partition GC. *Anal. Chem.* **1998,** *70*, 3828-3839.

35. *FFNSC 2.0 Flavors and Fragrances of Natural and Synthetic Compounds - Mass Spectral Database*. Chromaleont: Messina, Italy, 2007.

36. Costa, R.; De Fina, M. R.; Valentino, M. R.; Dugo, P.; Mondello, L. Reliable identification of terpenoids and related compounds by using linear retention indices interactively with mass spectrometry search. *Nat. Prod. Commun.* **2007,** *2*, 413-418.

37. Bicchi, C.; Artuffo, G.; D'Amato, A.; Manzin, V.; Galli, A.; Galli, M. Cyclodextrin derivatives in the GC separation of racemic mixtures of volatile compounds. Part 5. Heptakis 2,6-dimethyl-3-pentyl-β-cyclodextrin. *J. High Resolut. Chromatog.* **1992,** *15*, 710-714.

38. König, W. A.; Gehrcke, B.; Icheln, D.; Evers, P.; Dönnecke, J.; Wang, W. C. New selectively substituted cyclodextrins as stationary phases for the analysis of chiral constituents of essential oils. *J. High Resolut. Chromatog.* **1992,** *15*, 367-372.

39. Maas, B.; Dietrich, A.; Bartschat, D.; Mosandl, A. *tert*-Butyldimethylsilylated cyclodextrins: versatile chiral stationary phases in capillary gas chromatography. *J. Chromatogr. Sci.* **1995,** *33*, 223-228.

40. Bicchi, C.; D'Amato, A.; Manzin, V.; Galli, A.; Galli, M. Cyclodextrin derivatives in the gas chromatographic separation of racemic mixtures of volatile compounds. Part 10. 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β- and γ-cyclodextrins. *J. Chromatogr. A* **1996,** *742*, 161-173.

41. AMDIS (2012) Version 2.71. http://chemdata.nist.gov/dokuwiki/doku.php?id=chemdata:amdis (accessed July 2015).

42. Rubiolo, P.; Liberto, E.; Sgorbini, B.; Russo, R.; Veuthey, J. L.; Bicchi, C. Fast-GC-conventional quadrupole mass spectrometry in essential oil analysis. *J. Sep. Sci.* **2008,** *31*, 1074-1084.

43. Hardt, I.; König, W. A. Diluted versus undiluted cyclodextrin derivates in capillary gas chromatography and the effect of linear carrier gas velocity, column temperature, and length on enantiomer separation. *J. Microcolumn Sep.* **1993,** *5*, 35-40.

44. Bicchi, C.; Artuffo, G.; D'Amato, A.; Galli, A.; Galli, M. Cyclodextrin derivatives in GC separation of racemic mixtures of volatiles. Part 3. *Chirality* **1992,** *4*, 125-131.

45. Grosenick, H.; Schurig, V.; Costante, J.; Collet, A. Gas chromatographic enantiomer separation of bromochlorofluoromethane. *Tetrahedron: Asymmetry* **1995,** *6*, 87-88.

46. Lindstrom, M. Improved enantiomer separation using very short capillary columns coated with permethylated β-cyclodextrin. *J. High Res. Chromatog.* **1991,** *14*, 765-767.

47. Bicchi, C.; Blumberg, L.; Cagliero, C.; Cordero, C.; Rubiolo, P.; Liberto, E. Development of fast enantioselective gas-chromatographic analysis using gas-chromatographic method-translation software in routine essential oil analysis (lavender essential oil). *J. Chromatogr. A* **2010,** *1217*, 1530-1536.

48. Bicchi, C.; Liberto, E.; Cagliero, C.; Cordero, C.; Sgorbini, B.; Rubiolo, P. Conventional and narrow bore short capillary columns with cyclodextrin derivatives as chiral selectors to speed-up enantioselective gas chromatography and enantioselective gas chromatography-mass spectrometry analyses. *J. Chromatogr. A* **2008,** *1212*, 114-123.

49. Bicchi, C.; D'Amato, A.; Manzin, V. Derivatized cyclodextrins in enantiomer GC separation of volatiles. In *Flavours and Fragrances;* Swift, K. A. D., Ed.; The Royal Society of Chemistry: Cambridge, 1997; pp 57-69.

50. Kreis, P.; Dietrich, A.; Mosandl, A. Chiral compounds of essential oils. Part 18. On the authenticity assessment of the essential oil of *Rosmarinus officinalis* L. *Pharmazie* **1994,** *49*, 761-765.

51. Blumberg, L. M.; Klee, M. S. Optimal heating rate in gas chromatography. *J. Microcolumn Sep.* **2000,** *12*, 508-514.

52. Klee, M. S.; Blumberg, L. M. Theoretical and practical aspects of fast gas chromatography and method translation. *J. Chromatogr. Sci.* **2002,** *40*, 234-247.

53. GC Method Translation Software. www.chem.agilent.com (accessed July 2015).

54. Mondello, L.; Sciarrone, D.; Costa, R.; Dugo, G. The chiral compounds of citrus oils. In *Citrus oils, Composition, Advanced AnalyticalTtechniques, Contaminants and Biological Activity;* Dugo, G.; Mondello, L., Eds.; CRC Press: Boca Raton (FL), 2011; pp 349-404.

55. Blumberg, L. M. Theory of fast capillary gas chromatography - Part 3: Column performance vs. gas flow rate. *J. High Resolut. Chromatog.* **1999,** *22*, 403-413.

56. Cagliero, C.; Sgorbini, B.; Cordero, C.; Liberto, E.; Bicchi, C.; Rubiolo, P. Analytical strategies for multipurpose studies of a plant volatile fraction. In *Handbook of Chemical and Biological Plant Analytical Methods;* Hostettmann, K.; Stuppner, H.; Marston, A.; Chen, S., Eds.; Wiley: Chichester (UK), 2014; pp 1-20.

57. Manz, A.; Graber, N.; Widmer, H. M. Miniaturized total chemical analysis systems: a novel concept for chemical sensing. *Sens. Actuators, B* **1990,** *1*, 244-248.

58. Bicchi, C.; Cordero, C.; Liberto, E.; Sgorbini, B.; Rubiolo, P. Headspace Sampling in Flavor and Fragrance Field. In *Comprehensive Sampling and Sample Preparation;* Pawliszyn, J., Ed.; Elsevier, Academic Press: Oxford (UK), 2012; pp 1-25.

59. Rubiolo, P.; Sgorbini, B.; Liberto, E.; Cordero, C.; Bicchi, C. Analysis of the plant volatile fraction. In *The Chemistry and Biology of Volatiles* Herrmann, A., Ed.; Wiley: Chichester (UK), 2010; pp 50-93.

60. Sgorbini, B.; Bicchi, C.; Cagliero, C.; Cordero, C.; Liberto, E.; Rubiolo, P. Headspace sampling and gas chromatography: a successful combination to study the composition of a plant volatile fraction. In *Handbook of Chemical and Biological Plant Analytical Methods* Hostettmann, K.; Stuppner, H.; Marston, A.; Chen, S., Eds.; Wiley: Chichester (UK), 2014; pp 1-31.

61. Baltussen, E.; Cramers, C. A.; Sandra, P. J. F. Sorptive sample preparation - a review. *Anal. Bioanal. Chem.* **2002,** *373*, 3-22.

62. Cagliero, C.; Bicchi, C.; Cordero, C.; Rubiolo, P.; Sgorbini, B.; Liberto, E. Fast headspace-enantioselective GC-mass spectrometric-multivariate statistical method for routine authentication of flavoured fruit foods. *Food Chem.* **2012,** *132*, 1071-1079.

63. Bicchi, C.; Blumberg, L. M.; Rubiolo, P.; Cagliero, C. General retention parameters of chiral analytes in cyclodextrin gas chromatographic columns. *J Chromatogr. A* **2014,** *1340*, 121-127.