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Essential oils and volatiles: sample preparation and analysis. A review.†

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ABSTRACT: This article is a short overview of the state of the art in essential oil analysis. Several aspects of the analysis of essential oils and volatile fraction of vegetable matrices are here critically discussed. The following topics are dealt with: steam distillation, hydrodistillation and headspace sampling for sample preparation, and fast-GC, fast-GC–qMS analysis, enantioselective GC, multidimensional GC techniques and GC–isotopic ratio mass spectrometry (GC–IRMS) for analysis and quantitation.

Keywords: essential oils; volatile fraction; sample preparation; qualitative analysis; quantitative analysis

1. Introduction

An essential oil (EO) is internationally defined as the product obtained by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process without heating (for *Citrus* fruits) of a plant or of some parts of it.[1,2] This definition therefore allows us to include EOs within the more general framework of the volatile fraction of a vegetable matrix, which in its turn involves a range of other sampling approaches and/or techniques, producing samples that are representative (although almost always not comparable) of the volatiles characterizing a vegetable matrix, such as headspace, flavours, fragrances, aromas and extracts obtained by specific techniques. The term 'volatile fraction' in general defines a mixture of volatile compounds in a matrix of vegetable origin that can be sampled because of its ability to vapourize spontaneously and/or under suitable conditions or techniques.

These considerations are only apparently obvious since, unfortunately, in several articles, EO and headspace compositions have often (and erroneously) been compared directly or (even worse) have not even been distinguished. Although the compositions of an EO and that of the related headspace sampled by different techniques are sometimes similar, the areas (or percentages) of an analyte obtained with the two techniques cannot be compared, since they are obtained by entirely different approaches, which greatly influence the resulting quantitative composition and also, although to a lesser extent, the qualitative composition. This consideration clearly emerges from a comparison of the definitions of EO reported above and that of headspace, e.g. headspace sampling is a solvent-free technique aiming at sampling the gaseous or vapour phase in equilibrium (or not) with a solid or liquid matrix in order to characterize its composition.[3]

2. Sample Preparation

Quite a large number of conventional techniques may be used to sample the volatile fraction: vacuum distillation, steam distillation or hydrodistillation, solvent extraction off-line combined with distillation, simultaneous distillation–extraction (SDE), supercritical fluid extraction (SFE), and microwave-assisted extraction and hydrodistillation (MAE and MA-HD), static (S-HS), dynamic (D-HS) and high concentration capacity headspace (HCC-HS) sampling. It must here again be stressed that *only* the product obtained by hydrodistillation or steam distillation can be called 'essential oil' (of course, with the exception of cold expression for citrus fruits).

2.1. Steam Distillation and Hydrodistillation

An EO is classically obtained using equipment based on the circulatory distillation approach introduced by Clevenger.[4,5] In theory the recoveries of volatiles are quantitative for an infinite distillation time; thermal artefacts can be produced but they are accepted as a result of a traditional process. Apparatus and operation modes to obtain EO are well established and are described in several pharmacopoeias. Figure 1 shows a diagram of the apparatus reported in the *European Pharmacopoeia*.[2]

2.2. Headspace Sampling

Several factors have contributed to the remarkable development of headspace sampling (HS) over the last two decades: (a) hydrodistillation is time-consuming and cannot be combined on-line with analysis; (b) the ever-increasing demand for solvent-free (or solvent-less) sample preparation techniques, i.e. techniques in which the analyte(s) is isolated from a matrix without using a liquid solvent; (c) the exponential increase in the number of controls required in all fields, including flavours and fragrances, which cannot be satisfied by routine laboratories operating with

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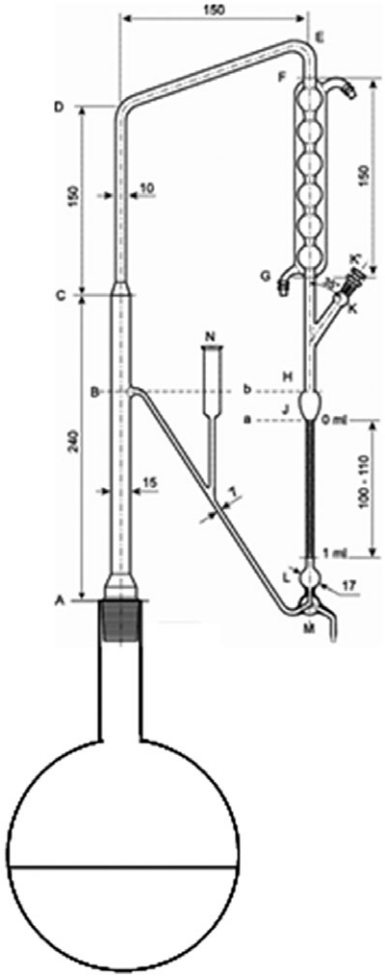

Figure 1. Clevenger circulatory distillation apparatus reported in the *European Pharmacopoeia*[2]

conventional techniques, and which make it indispensable to develop fully automatic analysis systems, in which sample preparation and analysis are integrated in a single step (better known under the acronym TAS, i.e. total analysis system).

Traditionally, HS sampling operates in either the static (S-HS) or the dynamic mode (D-HS), but interest in this technique has been renewed with the introduction, in the early 1990s, of an additional approach that acts as a bridge between S-HS and D-HS – high concentration capacity headspace techniques (HCC-HS).[6,7] This approach is based on either the static or the dynamic accumulation of volatile(s) on polymers operating in sorption and/or adsorption modes or, less frequently, on solvents. It has to be stressed that the recoveries with HCC-HS differ from one component to another, since these techniques are based on partition between more than two phases (matrix, headspace and polymer) and, in consequence, the sampled volatile fraction does not represent either the composition of the headspace or that of the essential oil. The HCC-HS techniques were immediately successful, mainly because they are as simple, fast, easy to automate and as reliable as S-HS, while at the same time showing analyte concentration factors that are very often comparable to those of D-HS. Several techniques based on this approach are now available, including: HS-solid-phase microextraction (HS–SPME),[8] in-tube sorptive extraction (INCAT, HS–SPDE),[9–11] headspace sorptive extraction (HSSE),[12,13] solid-phase aroma concentrate extraction (SPACE),[14] headspace liquid-phase microextraction (HS–LPME)[15,16] and large surface area HCC-HS sampling (MESI, MME, HS-STE).[17–19] HCC-HS, HS–SPME and S-HS, HCC-HS and D-HS techniques in the sample preparation of the plant volatile fraction, their advantages and limits, were recently reviewed by the authors' group.[6,7,20]

Cryogenic trapping is an effective dynamic approach to isolate the volatile fraction of a vegetable matrix which is very close in composition to headspace. Among others, two techniques based on cryogenic condensation of the stripped volatiles have successfully been applied to the flavour and fragrance field: cryogenic vacuum trapping, based on the stripping of the volatile fraction under vacuum,[21] and the Aquaspace® techniques, based on its stripping by humidified air.[22]

3. Analysis

Analysis of an EO usually involves the separation, identification and quantitative determination of its components. The volatility and polarity of EO components make capillary gas chromatography the technique of election for their analysis, because EOs in general are complex mixtures of components with similar physicochemical characteristics. An exhaustive EO separation can preferably be obtained by combining two different-polarity stationary phases. The most used apolar stationary phases in EO routine analysis are in general those based on methyl polysiloxanes (SE30, OV-1, OV 101, DB-1, HP-1, PS 347.5, etc.) and methylphenyl-polysiloxanes (SE-52, SE-54, DB-5, HP-5, PS-086, etc.) and polyethyleneglycol (PEG-20M, CW-20M, DB-Wax, etc.) as the polar phase. Identification is generally carried out either by chromatographic data (Kováts indices, linear retention indices, relative retention time, retention time locking), measurable with a universal detector such as FID or TCD, or by spectral data, mainly by mass spectrometry (GC–MS) or, better, by their combination, as required by IOFI[23] and scientific journals such as *Flavour and Fragrance Journal*[24] and the *Journal of Agricultural and Food Chemistry*.[25] Fourier transform infrared spectroscopy (FT-IR) has also been proposed as a detector for GC (GC–FT-IR)[26] but, in spite of its high complementarity to MS for component identification, after encouraging success when it was introduced in the mid-1980s, its use has decreased steadily.

Unlike what most researchers in the field think, several topics related to EO analysis require further in-depth investigation, e.g. fast-GC combined with FID and MS, reliable automatic identification approaches, multidimensional GC, enantioselective GC combined with FID (ES–GC) and/or MS (ES–GC–MS) and GC combined with isotopic ratio mass spectrometry (GC–IRMS). Lastly, special attention must be paid to quantitative analysis, both because its interest is constantly increasing and because its present status is not clear. These topics will be dealt with in more detail in later sections of this paper.

3.1. Fast-GC and Fast-GC–qMS EO Analysis

The demand for faster GC analysis is continually increasing. Although investigated since the early 1960s,[27,28] high-speed GC has only been used for routine EO analysis in the last few decades.

The easiest way to speed up a GC separation is to shorten the column length while keeping enough resolving power for the given separation problem. The various theoretical and practical aspects involved with fast GC (F-GC) have been reviewed by

Cramers *et al.*[27] Two approaches have been proposed to speed up the analysis of an EO. The first and most widely used one involves short columns with narrow internal diameter (i.d.; ≤0.1 mm), while the second approach adopts short capillary columns with conventional i.d. (SCC–GC).[29] F-GC, with narrow-bore columns, was first applied to EO analysis by Proot *et al.*[30] SCC–GC can successfully be applied to routine quantitative analysis of a medium-complexity EO (up to about 30 components), since the efficiency of capillary columns is frequently much higher than necessary. An effective separation can therefore be achieved even with a column of 5 m (instead of 30 m), whose efficiency (i.e. the number of theoretical plates) is four to eight times lower, but which enables the analysis time to be shortened by the same factor if combined with a suitable temperature programme. When efficiency is insufficient for good separation, its lack can be compensated by adopting a stationary phase with a suitable selectivity.[29]

The speed of a GC analysis was first objectively defined by Blumberg *et al.*[31] on the basis of the average peak width; this definition was integrated by Magni *et al.*,[32] who also involved analysis time and temperature rate. Today, it is generally accepted that a GC analysis is 'fast' when it runs in <10 min, with columns with i.d. 0.25–0.1 mm, length 5–15 m, temperature programmes at 20–60°C/min and peak widths in the range 0.5–5.0 s. On the other hand, the term 'ultra-fast GC' is used for analyses of 1 min or less, involving short (2–10 m) narrow-bore columns (0.1––0.05 mm i.d.) and temperature programmes >1°C/s, leading to peak widths of 0.05–0.2 s.[33]

The routine use of F-GC has been made possible by the introduction over the last 10–15 years of electronic pressure control of the mobile phase, detectors such as high-frequency FID and high-speed quadrupole (qMS) and time-of-flight mass-spectrometers (TOF) able to record reliable mass spectra from high speed peaks, as well as the development of software to facilitate the method revalidation that is necessary when conventional i.d. columns are replaced by narrow-bore columns.[34]

The use of F-GC for routine analysis has also been favoured by its compatibility with the recent generation of mass spectrometers with quadrupole analysers, i.e. the most popular MS detectors used in routine analysis, mainly due to their reliability and acceptable cost. F-GC–qMS is at present necessary in the EO field in view of the recent regulatory aspects, which have introduced stringent recommendations and that require an ever-increasing number of accurate quantitative analyses in the routine controls (e.g. suspected allergens in perfumes). The state of the art of the F-GC–qMS combination was exhaustively and critically reviewed by Mastovska *et al.*[35] F-GC–qMS in EO analysis was also recently investigated in depth by Rubiolo *et al.*[36] in a study dealing with separation, identification and quantitation of 10 components characteristic of peppermint EO. They showed that operating at a suitable scan speed in TIC mode (from 999 and 11111 amu/s) or at a suitable dwell time in SIM mode (0.5–100 ms), the results obtained with F-GC–qMS with a 10 m × 0.1 mm i.d. column combined with temperature programmes of 20–60°/min are fully comparable to those obtained by conventional GC–qMS, while reducing the analysis time by a factor better than 10 (from about 35 to 3–4 min). Scan speed and dwell time play a fundamental role because: (a) they influence the separation performance of the F-GC–qMS system when the temperature programme is increased, because they contribute to the correct definition of the peak shape through the number of points per peak that, in turn, conditions peak width and consequently separation power;

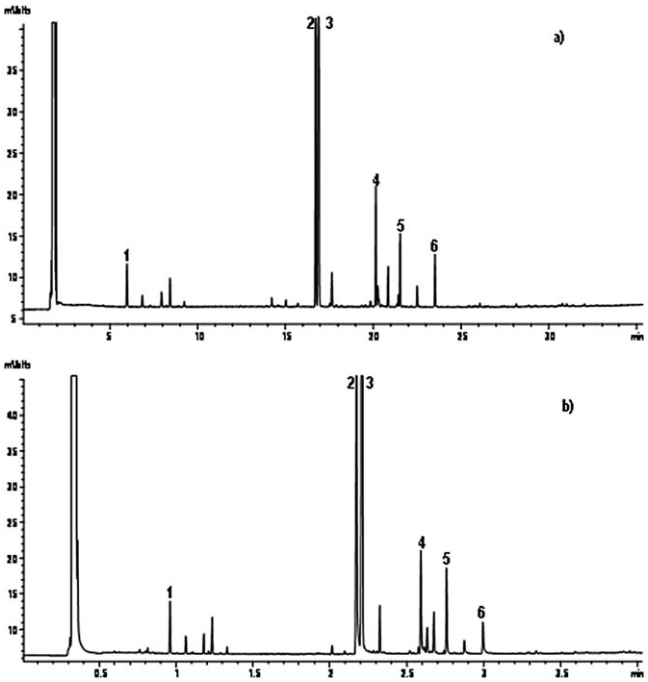

Figure 2. *Salvia sclarea* L. EO profiles by: (a) conventional GC–FID, column, Carbowax 20 M (50 m × 0.25 mm i.d., 0.25 μm film thickness). Temperature programmme, 60°C for 5 min, rising at 3°C/min to 220°C, then held for 30 min; carrier gas, hydrogen; flow rate, 2 ml/min; injection, split; ratio, 50/1; injection volume, 1 μl. (b) Fast GC–FID; column, Carbowax 20 M (10 m × 0.10 mm i.d., 0.10 μm film thickness). Temperature programme, 60°C for 0.61 min, rising at 41°C/min to 220°C, then held for 3.64 min; carrier gas, hydrogen; flow rate, 0.5 ml/min; injection, split; ratio, 200/1; injection volume, 0.5 μl. 1, β-myrcene; 2, linalool; 3, linalyl acetate; 4, α-terpineol; 5, geranyl acetate; 6, geraniol

and (b) they condition a correct peak area determination in both TIC and SIM–qMS modes at different F-GC speeds. Figure 2 reports the sage EO GC profiles analysed by conventional GC and F-GC. The same approaches have recently been used by Bicchi *et al.*[37] to speeding up enantioselective GC analysis (see below).

Component identification by GC–MS is mainly based on mass spectra, and the identification potential of GC is in general underestimated, not least because the identification capability of mass spectrometry when used as GC detector is exhaustive. Retention indices (RIs) are the most reliable and effective tool for analyte identification through GC data, in particular with EOs that very often consist of complex groups of mono- and sesquiterpenoids with very similar structures, polarities and volatilities and, in consequence, very similar mass spectra. Retention indices [better known as linear retention indices (LRIs)] were first introduced by Kováts[38] for isothermal analysis and then by Van den Dool *et al.*[39] for temperature programmed analysis. At present, most GC–MS software does not include LRIs as identification criteria, and only some of them report LRIs in the library as 'inactive' data appearing in the legend of each identification record proposed by the software, making them only useful for further or additional confirmation. On the contrary, the 'interactive' use of LRIs (i.e. their use as an 'active' identification parameter) can be very useful, since it offers a second independent tool to identify a compound, which can operate simultaneously and synergistically with MS spectra. Moreover, LRIs are based on a chemical property of the analyte, the nature of which is completely

different from its mass spectrum, i.e. the interactive LRI use of an analyte enables the orthogonal combination of its chromatographic interaction with a given chromatographic separation system or, better, with a given stationary phase with its MS fragmentation pattern.

As already mentioned, only a few commercially-available mass spectral library software packages includes retention index information to facilitate peak assignment.[40–46] The FFNSC MS Library system developed by Costa *et al.*[47] (FFNSC MS Library) is equipped with an interactive tool to calculate LRIs automatically, and incorporates LRIs as an active part of the match criteria in characterizing and identifying flavour and fragrance components, dramatically increasing the reliability of the MS response. A second software package actively using LRIs is automatic mass spectral deconvolution (AMDIS),[45] which is often used in combination with NIST Mass Spectral Libraries (both developed by the National Institute of Standards and Technology). This programme, too, enables mass spectra libraries containing retention index information to be used through a retention index matching window on raw data automatically converted into the AMDIS format.

Retention time locking (RTL) is a further approach for analyte identification, based on analyte retention behaviour for programmed temperature analysis.[48] The RTL approach involves adjustment of the carrier gas inlet pressure to provide an identical analyte retention time for a given compound in any system equipped with the same 'nominal' column. This approach is very useful to standardize retention times for routine analyses inside a laboratory, but it is more limited when data from different laboratories must be compared, unlike what happens with retention indices. A commercially-available RTL-based software package (Flavfid) is available; it can be combined with an additional mass spectral library when operating in GC–MS based on retention time-locked GC–FID and GC–MS data in the identification process.[49]

3.2. Enantioselective GC and Essential Oils

Chiral recognition of EO components was one of the most important milestones reached in essential oil analysis during recent decades. Enantiomer separation or excess (EE) or ratio (ER) determination is not only important because optical isomers can have different odours (e.g. limonene, carvone, etc.) but also because these isomers enable us to define the biosynthetic and geographical origins of the matrix investigated, and to investigate technological treatments undergone by, and/or authenticity of, most EOs.

Enantiomer separation in routine enantioselective GC (ES–GC) analysis has been made possible by the introduction of cyclodextrin derivatives (CDs) as chiral selectors. CDs were first introduced by Sibilska *et al.*[50] for packed columns and almost contemporarily applied to capillary columns by Juvancz *et al.*[51] and Schurig *et al.*[52] Moreover, Nowotny *et al.*[53] first proposed diluting CD derivatives in moderately polar polysiloxane (OV-1701) to improve their chromatographic properties and operative temperatures.[53] Since then, several hundreds of articles have been published dealing with the theory of ES–GC recognition with CDs, synthesis of new CD derivatives, their enantioselectivity and applications; many of these concern the flavour and fragrance field.[54–57]

A universal CD derivative suitable to provide the separation of most significant racemates in this field has not yet been found, mainly because of the intrinsic mechanism of chiral recognition in GC, which is based on a host–guest interaction of each enantiomer of a racemate with the CD selector, the separation depending on the rather small difference in the energy of interaction of each enantiomer with the CD chiral selector.[58,59] A laboratory must therefore have available at least two columns coated with different CD derivatives to enable separation of at least 80% of the most common racemates in the flavour and fragrance field. The most effective CD derivatives are nowadays those belonging to the so-called second generation, consisting of β-cyclodextrins substituted in position 6 (i.e. the narrow side of the CD) with a bulky group (*tert*-butyldimethylsilyl- or *tert*-hexyldimethylsilyl-) and with alkylated and acylated groups in positions 2 and 3 (mainly methyl, ethyl and acetyl) of its wide side. The effectiveness of CD derivatives as chiral selectors for ES–GC is clearly shown by the separation of the chiral test components developed in the authors' laboratory,[60] analysed on a 2,3-diethyl-6-*t*-butyl-dimethyl-silyl-β-cyclodextrin (2,3-DE6TBDMS-β-CD) diluted in PS-086, reported in Figure 3.

Chiral recognition of marker compounds in complex real-world samples often requires a two-dimensional (2D) approach. Two complementary but distinct strategies can therefore be adopted:

1. The first and most popular strategy is based on a second dimension *in separation*. Chiral recognition is generally carried out either by conventional heart-cut GC–GC,[61–64] or by comprehensive GC×GC (see next paragraph) when very complex samples or/and a very large number of components must be investigated simultaneously.[65,66]
2. The second approach is based on a second dimension *in identification*. In this case, an enantiomer is selectively isolated in the chromatogram by spectroscopic detection (usually MS) in single- or multiple-ion monitoring-MS (SIM–MS) through a careful choice of suitable diagnostic ions characterizing the investigated enantiomers. Since MS is not a selective chiral probe that discriminates between optical isomers, and since it gives indistinguishable spectra, an enantiomer can only be identified unequivocally through its LRI, determined on a column coated with a chiral selector suitable to separate it. In ES–GC–MS, mass spectra (or diagnostic ion monitoring) are therefore used to locate the enantiomers in the chromatogram, and LRIs to identify them. On the basis of this approach, construction of a MS library specific for the identification of optically-active compounds in the flavour and fragrance field, using 'active' LRIs in parallel to MS spectra, was recently described.[67]

3.3. Multidimensional GC Techniques

The most exhaustive definition of multidimensional (MD) separation was introduced by Giddings in 1987, who defined MD as 'an orthogonal two-column separation, with complete transfer of solute from the separation system 1 (column 1) to the separation system 2 (column 2), such that the separation performance from each system (column) is preserved'.[68] Two main approaches are currently adopted in the GC analysis of highly complex volatile fractions of plant matrices: the so-called heart-cut GC–GC and two dimensional comprehensive GC (GC×GC), or more simply GC×GC. Heart-cut GC–GC is a technique where a fraction (or a very few fractions) eluting from a first column are on-line and directly transferred to the second column for further separations. The transfer is obtained by dedicated time-programmable inter-

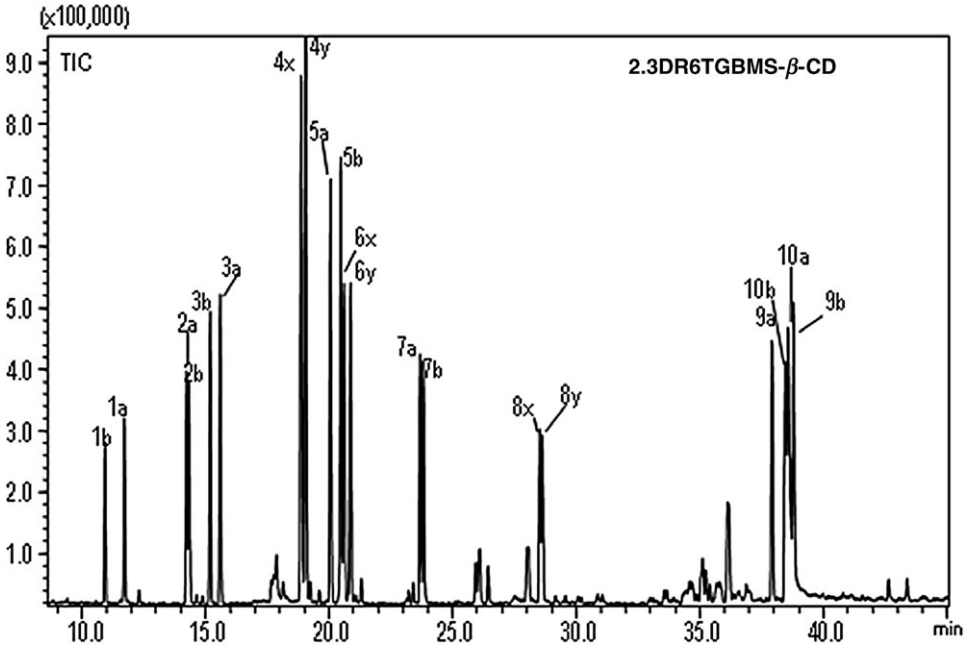

Figure 3. Chiral test profiles carried out on the 30% 2,3-DE6TBDMS-β-CD/PS-086 column (25 m × 0.25 mm i.d., 0.15 μm film thickness). Temperature programme, 40°C for 5 min, rising at 2°C/min to 220°C, then held for 30 min; carrier gas, helium; flow-rate, 2 ml/min; injection, split; ratio, 50/1; injection volume, 1 μl. 1, limonene; 2, 2-octanol; 3, camphor; 4, isobornyl acetate; 5, linalyl acetate; 6, 2-methyl-(3*Z*)-hexenyl butyrate; 7, menthol; 8, hydroxycitronellal; 9, γ-decalactone; 10, δ-dodecalactone. a, *(R)* enantiomer; b, *(S)* enantiomer; *x* and *y*, enantiomer configuration not assigned

faces.[69,70] This technique therefore allows us to analyse only critical pairs or selected groups of compounds or markers on the second dimension, and it is widely used in particular for ES–GC. Figure 4 shows the heart-cut MDGC pattern of *Lavandula angustifolia* P. Mill EO (for experimental conditions, see caption to the figure).

However, heart-cut GC–GC does not fully meet Giddings' definition, whereby *each peak* eluting from the first dimension has to be automatically re-injected into the second dimension. GC×GC was introduced by Liu *et al.*[71] to meet this condition, which is achieved by a peak modulator. In GC×GC each peak eluting from the first dimension is 'cut' into thin slices during a fixed time (4–8 s) by cryogenic focusing. Each slice is then on-line injected into the second column where it must be analysed in the same time as that of modulation. As a consequence, a conventional GC×GC system generally consists of a 0.25 mm i.d. column producing peaks at least 6–8 s width combined with a short, narrow-bore column (<1.5–2 m) to analyse each peak slice so that an analysis for each modulation period is produced. GC×GC, in particular when combined with MS, is the most powerful separation system now available. The separation power of GC×GC is evident when complex EOs are analysed. Figure 5a reports the one-dimensional (1D) cGC pattern of the EO of the non-toxic variety of *Ferula communis* L. and Figure 5b shows the contour plot of the same EO analysed by. The number of peaks detected is dramatically different: 115 peaks with 1D-cGC, and 532 with GC×GC.

3.4. GC–Isotopic Ratio Mass Spectrometry (GC–IRMS)

The ratio between stable isotopes is a widely-used parameter in several fields, including biochemistry, food and drug research and in origin assignment and in authenticity control. In an on-line coupled GC–IRMS system, analytes eluting from the GC column are combusted to carbon dioxide in a specially designed oven and directly introduced into an isotope ratio mass spectrometer (ref. [72] and references cited therein). The system is tuned to measure the mass ions 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$, $^{12}C^{16}O^{17}O_2$) and 46 ($^{12}C^{16}O^{18}O$) simultaneously in the nmole range and with high precision (≤0.3‰). The peak ratio is given by the area ratio of two isotope peaks and compared to a standard value through the following equation:

$$\delta = (R_{SA}/R_{ST} - 1) \times 1000$$

where R_{SA} is the isotope ratio of the sample and R_{ST} that of the standard; δ-C^{13} is given in parts per thousand.

The δ-C^{13} value is particularly significant when calculated on the enantiomer(s) separated by ES–GC and characteristic of the plant matrix.[72] ES–GC results alone are not always sufficient to detect adulteration of an EO, in particular with racemates of natural origin or when racemization is a consequence of processing and/or storage of the original product, or else when the EO is blended with a synthetic enantiomer. On the other hand, ES–GC combined with IRMS is highly effective to prove EO authenticity, since enantiomers from the same natural source are expected to have the same δ-C^{13}, even with partially racemized chiral molecules, since, in the same organism, racemic compounds are in general formed through the same biochemical pathways. ES–GC–IRMS or, even better, ES–MDGC–IRMS, is the most effective tool to detect adulteration of EOs containing optically-active components with the corresponding synthetic products or racemates.

3.5. Quantitative Analysis

The demand for quantitative data in the EO field is mainly due to their increased economic importance and to the continual

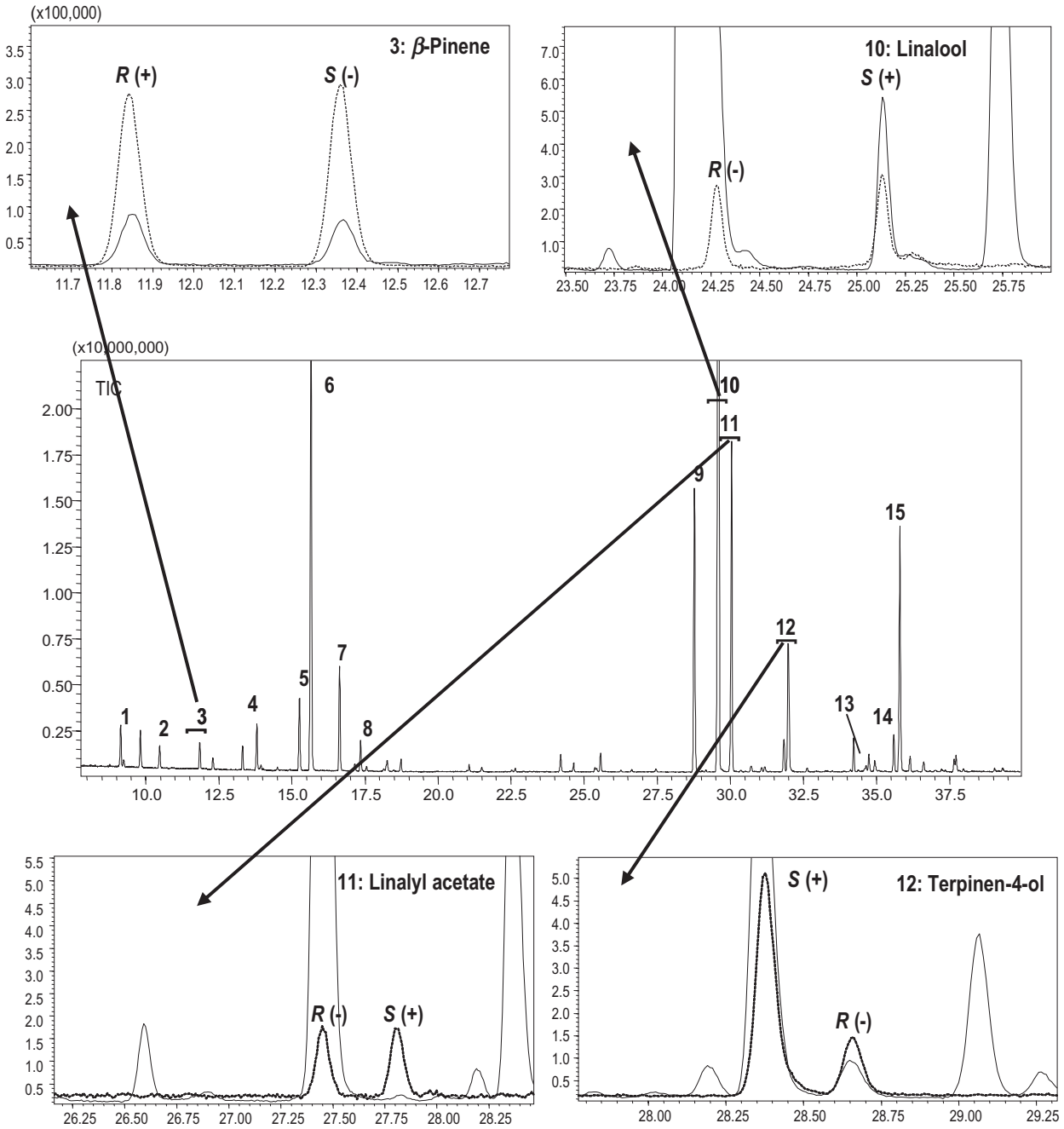

Figure 4. Heart-cut MDGC analysis of *Lavandula angustifolia* P. Mill EO. 1st column, CW (60 m × 0.25 mm i.d., 0.25 mm film thickness); 2nd column, 2,3-DE6TBDMS-β-CD columns (25 m × 0.25 mm i.d., 0.15 µm film thickness); temperature programme, 1st column 50°C, rising at 3°C/min to 220°C; 2nd column, 60°C, rising at 2°C/min to 180°C. Reference standards in dashed lines. Peak identification: 1, α-pinene; 2, camphene; 3, β-pinene; 4, myrcene; 5, limonene; 6, 1,8-cineole; 7, *cis*-β-ocimene; 8, *trans*-β-ocimene; 9, camphor; 10, linalool; 11, linalyl acetate; 12, terpinen-4-ol; 13, lavandulol; 14, α-terpineol; 15, borneol

increase in controls to verify quality, safety and biological activity. The quantitative aspects of EO analysis are not easy to deal with, not only because component identification is in general a priority to the detriment of quantitation, but also because the approach to it is often ambiguous. This topic has recently been critically discussed by Bicchi *et al.*[73] The quantitative composition of most EOs is very often reported in the literature in terms of relative percentage abundances, although this approach can unfortunately only give an approximate indication of the ratio between components in the sample under investigation.

There can be no single absolute approach to quantitation, because of the complexity of EOs and the different methods to quantify their components. To the best of the authors' knowledge, the most widely-used approaches with EOs are: (a) relative percentage abundance; (b) internal standard normalized percentage abundance, and quality characterization by statistical elaboration of the GC profile assumed as a parameter representative (fingerprint) of the sample investigated within a set; (c) 'absolute' or true quantitation of one or more components; and (d) quantitation by a validated method.

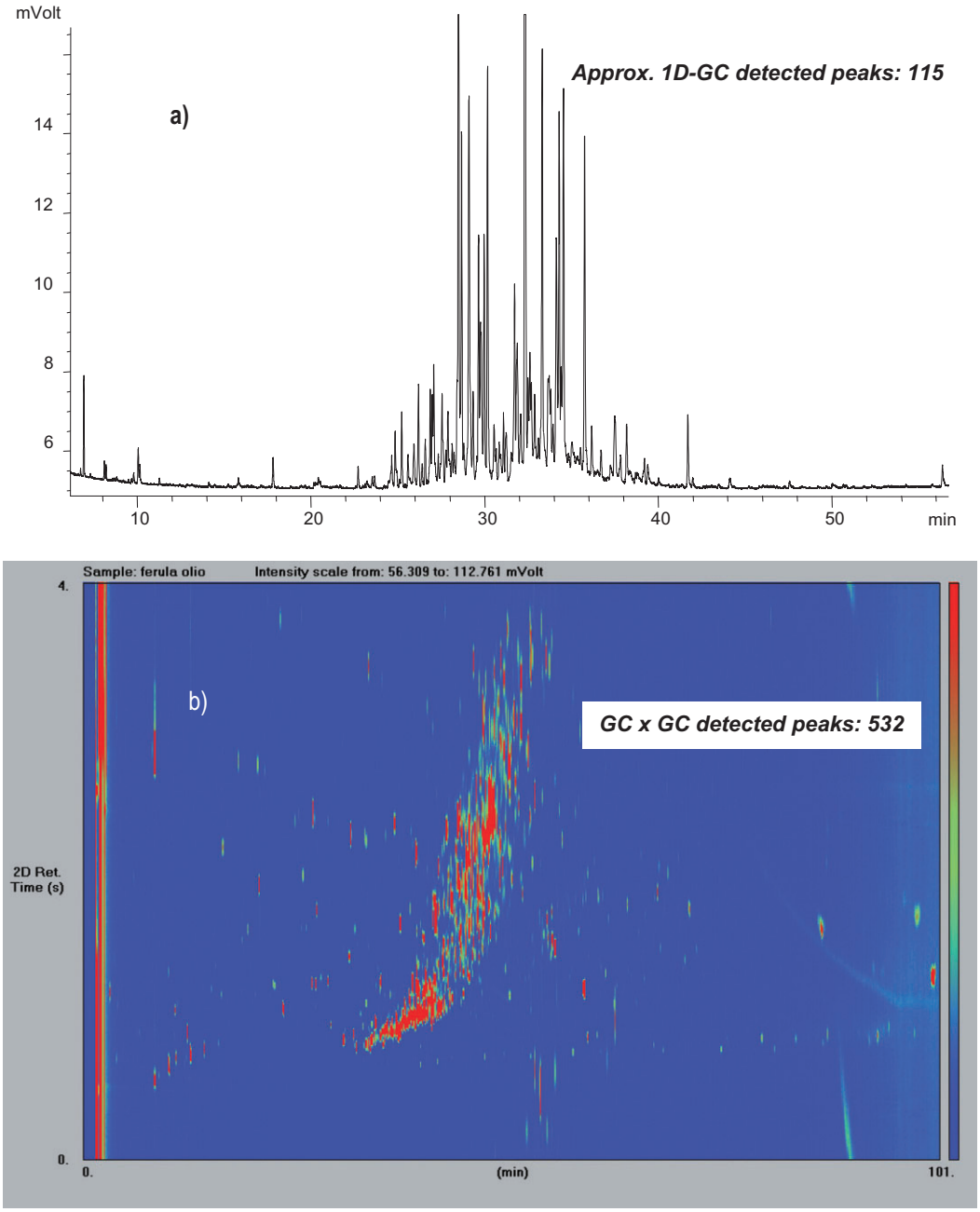

Figure 5. (a) 1D cGC pattern of the EO of the non-toxic variety of *Ferula communis* L. (b) Contour plot of the same EO, analysed by GC×GC. Number of peaks detected: 1D-cGC, 115; GC×GC, 532

It is well known that a quantitative analysis consists of two main steps: sample preparation and the analysis itself. For sample preparation, some basic indications must be followed to obtain reliable quantitative results, whatever method is used to sample the volatile fraction (steam distillation, hydrodistillation, mechanical procedures or HCC-HS techniques): (a) the variability of a plant matrix requires careful standardization, both during collection and in sample preparation; (b) a suitable number of samples must be analysed to obtain a representative composition of the volatile fraction of the species investigated (i.e. the averaged composition from at least three samples from distinct different populations).

As already mentioned, relative percentage abundance is the most commonly-used approach, although to date it is often incorrectly used, in particular to compare compositions within a set of EO samples from the same species. Relative percentage abundance results are correct only when used to evaluate relative component ratios within the same sample. When a group of EOs must be compared, raw data must first be normalized vs. an internal standard (or at least vs. an external standard if an automatic injector is available) and percentage abundance *must* be calculated vs. a fixed number of selected components taken as markers, usually common to all investigated samples. Moreover, normalized percentage data can also be used for profile analysis, i.e. statistical processing enabling us to discriminate or classify samples within a set, through the abundances of a given number of markers characteristic of the EO under investigation. The most widely-used approach is multivariate analysis, in particular

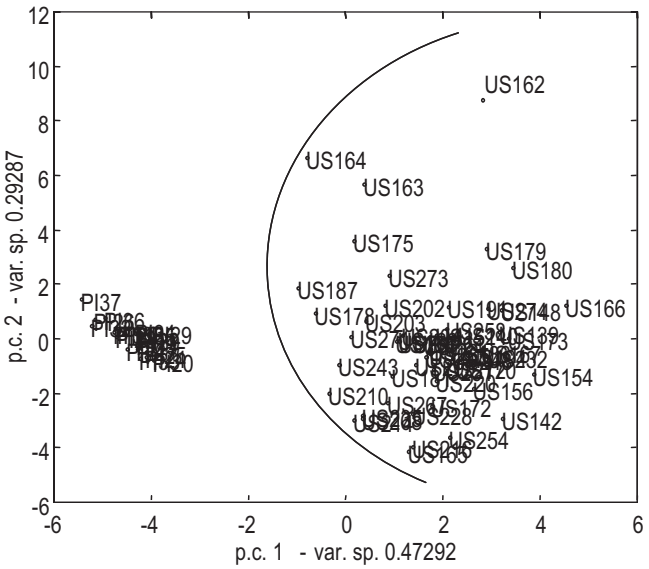

Figure 6. PCA scatterplots of the scores of the peppermint EOs. P, Italian EOs; US, US EOs

principal component analysis (PCA), a method that can explain the differences within a set of samples characterized by a suitable number of components (variables) through the linear combination of those explaining most of the variability.[74] Figure 6 reports the PCA discrimination for quality control of 93 peppermint EOs of different origins (Italy and USA), using normalized percentage abundances of 28 components present in all the samples after their standardization and/or normalization to a single internal standard. The so-called 'Italian peppermint' EOs can be distinguished perfectly from those originating from the US, with a total variability explained by the two principal components of 78%.

Quantitation can nowadays be achieved using several GC detectors, the most popular being FID and MS. The most important detector characteristics, when normalized percentage abundance is adopted, are that linearity and analyte response factors be as close as possible to one. The thermo-conductivity detector (TCD) is known to give a constant response for all sorts of structure and can be used successfully in combination with conventional capillary GC in EO quality control, provided that high sensitivity is not necessary.[75] On the other hand, FID is the most popular GC detector, being universal, highly sensitive and robust, but it is well known that its response factors with some compounds are not always close to one, e.g. for some esters it can be as high as 1.6, compared to *n*-nonane, taken as internal standard.[76] The use of MS as detector for quantitation is continually increasing, since at the same time it also provides component identification, although it can only be used in SIM mode and not for normalized percentage abundance, because ion abundances depend on analyte structure, which is mass-sensitive.

In any case, a normalized percentage abundance, i.e. a 'quantitative' comparison of GC profiles, is not sufficient to solve all quantitation problems concerning an EO. In some cases, an EO must be characterized by determining the concentration or absolute amount of one (or more) of its markers. A 'true' or an 'absolute' quantitation is therefore necessary; the investigated analyte(s) must be quantified after standardization of the chromatographic results by an internal (or external) standard, through a calibration curve constructed in the operative range of concentrations with the pure standard of the analyte under investigation. Since pure standards are not always commercially available, or are difficult to isolate, an accepted compromise is to use compounds belonging to the same class (hydrocarbons, aldehydes, alcohols, esters, etc.) of the analyte investigated, having a structure as similar as possible to it.[76]

Validated methods for specific biologically-active EO components are increasingly necessary, in particular for EOs used in the pharmaceutical industry. Dedicated guidelines established by the international regulatory bodies and committees (Eurachem, CITAC, IUPAC, etc.)[77–79] must be followed to validate a method, evaluating its performance through parameters such as selectivity, specificity, linearity in the working range, repeatability, precision, intermediate precision, accuracy/trueness and uncertainty assessment. The number of experiments required to develop a validated method meeting all these parameters, and the time, and as a consequence the costs entailed, limits their development to those applications where they are expressly required, and to a small number of analytes. It is unrealistic to think of the absolute quantitation of all EO components, not least in consideration of their complexity.

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