New trends in the analysis of the volatile fraction of matrices of vegetable origin. a short overview. A review

NEW TRENDS IN THE ANALYSIS OF THE VOLATILE FRACTION OF MATRICES OF VEGETABLE ORIGIN: A SHORT OVERVIEW

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Abstract: The term volatile fraction is a framework including a range of approaches and/or techniques, which produce samples that, while they may have different compositions, are representative of the volatiles characterising a vegetable matrix, e.g. headspace, essential oils, flavours, fragrances, aromas and extracts prepared through specific techniques. Its study requires analytical methods and technologies able not only to evaluate its composition exhaustively but also to monitor variations of its profile and to detect trace components characterizing the plant investigated.
The strategies of analysis have strongly changed over the last 15 – 20 years also because of the introduction of a group of new approaches, in particular:
- solventless sample preparation techniques, and, in particular, headspace sampling by High Concentration Capacity Headspace Techniques (HCC-HS e.g. HS-SPME, HSSE, STE, SE-HSSE);
- fast GC and fast-GC-fast-MS, and enantioselective GC (ES-GC) and ES-GC-MS analysis;
- new analytical techniques such as comprehensive GC (GCxGC);
- new operative strategies based on approaches (e.g. Analytical Decision Makers (ADM)) or technologies (e.g. Total Analysis Systems (TAS)) in some cases developed for other fields (environmental pollution, oils and fats, petrol, etc.) and applied to plant analysis;
- data elaboration strategies producing a higher level of information (metabolite profiling).
This article is a short overview on 1) the advancement of analytical techniques and how they can influence strategies and approaches to study the plant volatile fraction, and 2) enantiomer GC and GC-MS separation using cyclodextrins as chiral selectors

Introduction

The plant volatile fraction is an important marker diagnostic not only of plant origin and quality[1], but also of the compositions of other even-non-volatile fractions [2]. The term volatile fraction is a framework involving a range of approaches and/or techniques (headspace, essential oils, flavours, fragrances, aromas and extracts prepared through specific techniques) producing samples that, while they may have different compositions, are representative of the volatiles characterising a vegetable matrix. The study of the volatile fraction requires analytical methods and technologies able not only to evaluate its composition exhaustively but also to monitor its variations and to detect trace components characterizing the plant investigated. As a consequence, analytical set-up based on sampling techniques where recovery over time of the components of interest is maximized and analytical techniques reducing analysis time to a minimum are necessary to satisfy the ever increasing request of control analysis of plants to be applied in the food, cosmetic and pharmaceutical fields [2, 3].
The overwhelming evolution that has taken place in analysis over the last decades has strongly influenced the strategy to be adopted in each of the steps of an analytical procedure,

i.e. sample preparation, analysis and data elaboration. The present trend is to develop fast and automatic analysis methods and in particular Total Analysis System (better known as TAS), i.e. systems where the above steps are on-line combined in a single step.
These strategies have not only involved the development of new powerful techniques and tools for each step of the analytical process but also of approaches borrowed from other analytical fields and, when necessary, from other disciplines (e.g. economy or statistics).
This article is a very short overview on the evolution of sample preparation, analysis and data elaboration when applied to the study of the volatile fraction; in addition, a special paragraph is dedicated to enantioselective GC.

Sample preparation

The need of techniques for TAS systems has favored the development not only of highly-effective, high-speed, but also, and mainly, simple and easy to automate sample preparation procedures. In addition to highly effective conventional extraction (SFE, MAE, ASE etc.) and solventless clean-up techniques (e.g. SPE), this need has remarkably contributed to renew the interest for headspace (HS) sampling, also because of the possibility of these techniques to be on-line combined with GC or GC-MS [2-4]. Most of the new HS techniques belong to the so-called High Concentration Capacity Headspace Techniques (HCC-HS e.g. HS-SPME, HSSE, STE, SE-HSSE, etc.), i.e. a group of HS sampling methods based on the static accumulation of the analytes on a polymeric phase mainly by sorption and/or adsorption. HCC-HS techniques combines the advantages of static and dynamic HS, since they are as rapid, simple and as easy to automate as static HS and achieve analyte concentration factors comparable to those of dynamic HS. The HCC-HS approach was started by Zhang and Pawliszyn in 1993 [5] with the application of solid phase microextraction (SPME) to headspace sampling. One of the most complex aspects with headspace sampling (and in particular with HCC-HS) is quantitation that in general is run by three methods: Standard Addition (SA), Stable Isotope Dilution Assay (SIDA) or Multiple Headspace Extraction (MHE). SA and SIDA are mainly effective for liquid and liquid-suspended samples while MHE is preferable for solid samples since it avoids many drawbacks related to the matrix effect [6,7]. The reliability of routine quantitation by HCC-HS techniques is assured by the possibility to control the consistency of performance over time of the accumulating polymer by an equilibrium in-fibre internal standardization introduced by Pawliszyn's group for SPME[8,9] and easy to extend to the other HCC-HS techniques [7,10].

Separation and analysis

Gas chromatography in combination with mass spectrometry (GC-MS) is the method of choice for the analysis of volatile fractions. GC-MS nowadays evolves toward the speeding up of separation and detection steps, that is with approaches and instrumentation suitable to reduce analysis time to a minimum while keeping separation and producing reliable qualitative and quantitative results [11]. This goal has been achieved with short narrow bore columns and modern GC instrumentation provided with automatic injectors, electronic flow control, oven with high precision temperature and temperature rate controls, detector with high sensitivity, electronic stability (low signal-to-noise ratio) and frequency of signal acquisition. An important contribution to F-GC in routine analysis was the method translation approach introduced by Klee and Blumberg [12], which makes it possible to find the optimal separation/speed trade-off for a conventional GC method and to derive fast GC conditions from it automatically [13].
The GC stationary phases adopted in the flavour and fragrance field are well established; the most used of them are apolar polydimethylsiloxane as such (OV-1, DB-1, HP-1, Mega 1, Se-30 etc.) or with 5 % of phenyl groups (DB-5, HP-5, SE-52, Mega 5, etc.) and moderately

polar polyethylene glycols (DB-Wax, HP-wax, CW-20M, Megawax etc.). These phases have become references mainly because samples are often complex and mass spectra of compounds belonging to the same class or groups (e.g. monoterpenes or sesquiterpenes) are very similar, thus making chromatographic data (in particular Linear Retention Indices (LRI)) indispensable for a correct identification. New perspectives have been opened by the introduction of the ionic liquids as stationary phases for GC because of their selectivity towards specific chemical classes completely different from that of the above phases [14-16]. Room temperature ionic liquids are low volatility and highly stable organic non-molecular solvents liquid at 20°C consisting of an organic cation containing nitrogen or phosphorus (i.e. alkyl imidazolium, phosphonium) and an organic or inorganic anion.
The success of Fast-GC has also required further developments of mass detectors with both quadrupole (qMS) or time of flight (TOF-MS) analysers to enable them to acquire a number of reliable spectra per second compatible with the peak width (ranging from 5 to 0.2 seconds) deriving from Fast-GC to afford safe automatic analyte identifications and/or SIM-MS quantitation.
Figure 1 reports the conventional and translated fast GC profiles of juniper essential oil (*Juniperus communis* L.), showing that the two GC profiles are fully superimposable while analysis time decreases from about 60 minutes to about 20 minutes (for analysis conditions see captions to the figure).

Data elaboration – Over the last ten to fifteen years the elaboration of GC-MS data has strongly progressed not only because of new powerful software and availability of exhaustive and specialized libraries and collection of spectra, but also because of the introduction in routine of new tools such as the MS spectra deconvolution of co-eluting peaks and the interactive combination of chromatographic Linear Retention Indices (LRI) and MS data very helpful to produce highly effective component identification [17,18].
The recent progress in chemometrics and software for statistical elaboration have extended the range of information obtainable from a conventional analysis making the chromatographic profile (or MS profiles), defined by the abundances (better if normalised percentage data) of a selected number of markers, a characteristic of the investigated matrix, i.e. a further distinctive parameter to characterize a sample. Figure 2 reports the PCA scatterplot for supposed chemotype discrimination (provisorily defined as α-pinene, sabinene and intermediate types) through the essential oil GC profiles of 60 samples of juniper twigs from Norway (*Juniperus communis* L.).The importance of the analytical profile to characterize a sample as such or to discriminate it within a set is even higher with two dimensional chromatographic techniques. New fingerprinting approaches specific to GCxGC have recently shown its potential in sample comparisons and correlations, and its ability to locate compounds whose distribution can be correlated to sensory properties and geographical origin, or to monitor the effect of technological treatments on different classes of compounds [19-21].

Enantioselective GC with cyclodextrins as chiral selector - Enantiomer separation and enantiomeric excess (e.e.) or ratio (e.r.) determination have continuously gained in interest not only because of possible difference in enantiomer biological properties (*e.g.* different odours, specificity with insects, *etc.*) but also because of the contribution that chiral recognition can give to define the biosynthetic pathway of a component in vegetable matrix, to confirm its geographical origin and/or the technological treatments it has undergone, and its authenticity or possible frauds.
Derivatized cyclodextrins (CDs) are the most popular chiral selectors for enantioselective GC (ES-GC) in the flavour and fragrance field. They were first applied by Sibilska *et al.* in 1983

[22] for packed GC columns and then by Juvancz et al. in 1987 [23] and Schurig et al. [24] for capillary GC columns. In 1989, Nowotny et al. [25] proposed to dilute CD in moderately polar polysiloxane (OV-1701) to improve their chromatographic properties and extend the range of their operative temperatures.

Enantiomer separation with CDs is based on energetically different host-guest interaction that each enantiomer of a racemate establishes with the cyclodextrin chiral selector [26,27]. The most effective CDs nowadays available are those based on β-cyclodextrins substituted in position 6 *(i.e.* the CD narrow side) with a bulky group (*tert*-butyldimethylsilyl- or *tert*-hexyldimethylsilyl-) [28] and with "small" alkyl or acyl groups in positions 2 and 3 (mainly methyl, ethyl and acetyl).

Chiral recognition of components in complex samples in the flavour and fragrance field often need a two-dimensional approach. Two complementary but distinct strategies can be adopted:

a) the first and best known of them is based on a second dimension *in separation*. (conventional heart-cut GC-GC [29,30], or GCxGC [31,32]);

b) the second one uses mass spectrometry (MS) as a second dimension *in detection*. [33,34]

Advantages and limits of both approaches are well known, although recently mass spectrometry as a second dimension in detection has gained further interest because of the role it can play in speeding-up ES-GC analysis. Fast ES-GC analysis can successfully be achieved using: (a) short conventional or narrow bore columns; (b) mass spectrometry as detector to locate the enantiomers in the chromatograms and linear retention indices (I^Ts) to identify them [35]; and (c) temperature rates up to 10°C/min.

Narrow bore columns even shorter than the conventional 10 m long (e.g. 5 and 2 m) if suitably combined with SIM-mass spectrometry can shorten the analysis time by a factor of at least three compared to conventional ES-GC analysis while keeping the enantiomer resolution of chiral markers to the baseline ($r \geq 1.5$), i.e. the minimum required for a correct e.e. and e.r. determination [34]. Comparable results can be obtained by a complementary approach that is based on the optimization of the chromatographic separation and consists in finding the best trade-off between resolution of the most critical peak pairs and analysis time by optimizing the critical parameters of the GC method translation approach (void time (t_M), normalized temperature rate (r), efficiency-optimized flow (EOF) and speed optimal flow (SOF)) with the conventional analysis and then automatically translate them to the corresponding narrow bore columns [35]. Figure 3 reports conventional and fast HS-SPME-ES-GC-MS profiles of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway (for sampling and analysis conditions see captions to the figure). Table 2 reports the range of percent abundance of the (+)-enantiomer calculated as percentage of the sum of the areas of the two enantiomers of each marker for the three different juniper supposed chemotypes (*Juniperus communis* L.) mentioned in figure 2 determined by HS-SPME-ES-GC-MS analysis on 60 twig samples from Norway (for analysis conditions see caption to figure 3).

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Table 1 List of the acronyms:

ADM	Analytical Decision Maker
ASE	Accelerated Solvent Extraction
ES-GC	Enantioselective Gas Chromatography
ES-GC-MS	Enantioselective Gas Chromatography – Mass Spectrometry
EOF	Efficiency-optimized flow
GC	Gas Chromatography
GC-GC	Heart-cut two dimension Gas Chromatography
GCxGC	Comprehensive Gas Chromatography
GC-MS	Gas Chromatography – Mass spectrometry
HCC-HS	High Concentration Capacity Headspace Techniques
HS	Headspace
HS-SPME	Headspace Solid Phase Microextraction
HSSE	Headspace Sorptive Extraction
LRI	Linear Retention Indices
MAE	Microwave Assisted Extraction
MHE	Multiple Headspace Extraction
SA	Standard Addition
SE-HSSE	Solvent Enhanced - Headspace Sorptive Extraction
SFE	Supercritical Fluid Extraction
SIDA	Stable Isotope Dilution Assay
SIM-MS	Single Ion Monitoring Mass Spectrometry
SOF	Speed optimal flow
SPE	Solid Phase Extraction
STE	Sorptive Tape Extraction
TAS	Total Analysis Systems

Table 2 – Range of percent abundance of (+)-enantiomer of the markers of three different juniper supposed chemotypes (*Juniperus communis* L.) determined by HS-SPME-ES-GC-MS analysis on 60 samples from Norway (for analysis conditions see caption to figure 3)

Compounds	***Supposed chemotype***		
	α-Pinene	***Sabinene***	***Intermediate***
(+)-α Pinene	55-99%	46-67%	53-54%
(+)-β-Pinene	14-46%	29-55%	13-17%
(+)-Sabinene	69-99%	98-99%	98-99%
(+)-Limonene	91-97%	91-98%	70-95%
(+)-β-Phellandrene	1-4%	1-31%	1-2%
(+)-Terpinen-4-ol	68-99%	83-91%	81-87%

Captions to figure

Figure 1: GC/MS profiles of the essential oil of a juniper (*Juniperus communis* L.) twig sample from Norway

Analysis conditions: Instrumentation: Shimadzu QP2010 GC-MS system, provided with a Shimadzu AOC 5000 autosampler, and a Shimadzu GC-MS Solution 2.51 software. 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.

a) *Conventional-GC-MS*: Column MEGA 5 (25m x 0.25 mm x 0.25 µm). Temp. progr.: from 40°C (1 min) to 250°C (5 min) at 3 °C/min.; b) *Fast-GC-MS*: Column MEGA 5 (10 m x 0.10 mm x 0.10 µm). Temp. progr.: from 40°C (1.46 min) to 250°C (1.46 min) at 10.25 °C/min.

Peak identification: 1. α-thujene, 2. α-pinene, 3. β-pinene+sabinene, 4. myrcene, 5. α-terpinene, 6. p-cymene, 7. limonene + β-phellandrene, 8. α-terpinene, 9. terpinolene, 10. terpinen-4-ol, 11. α-cubebene, 12. α-copaene, 13. β-elemene, 14. β-caryophyllene, 15. germacrene D, 16. δ-cadinene, 17.germacrene B, 18. germacran-D-4-ol + spathulenol, 19. α-cadinene. Internal standard: undecane.

Figure 2 PCA supposed chemotype discrimination of 60 samples of juniper twig essential oils from Norway (*Juniperus communis* L.) analysed by fast GC-FID (for analysis conditions se caption to figure 1).

Figure 3 HS-SPME-ES-GC-MS profiles of the volatile fraction of a juniper (*Juniperus communis* L.) twig sample from Norway

HS-SPME 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: 10 min, temperature: 50°C.

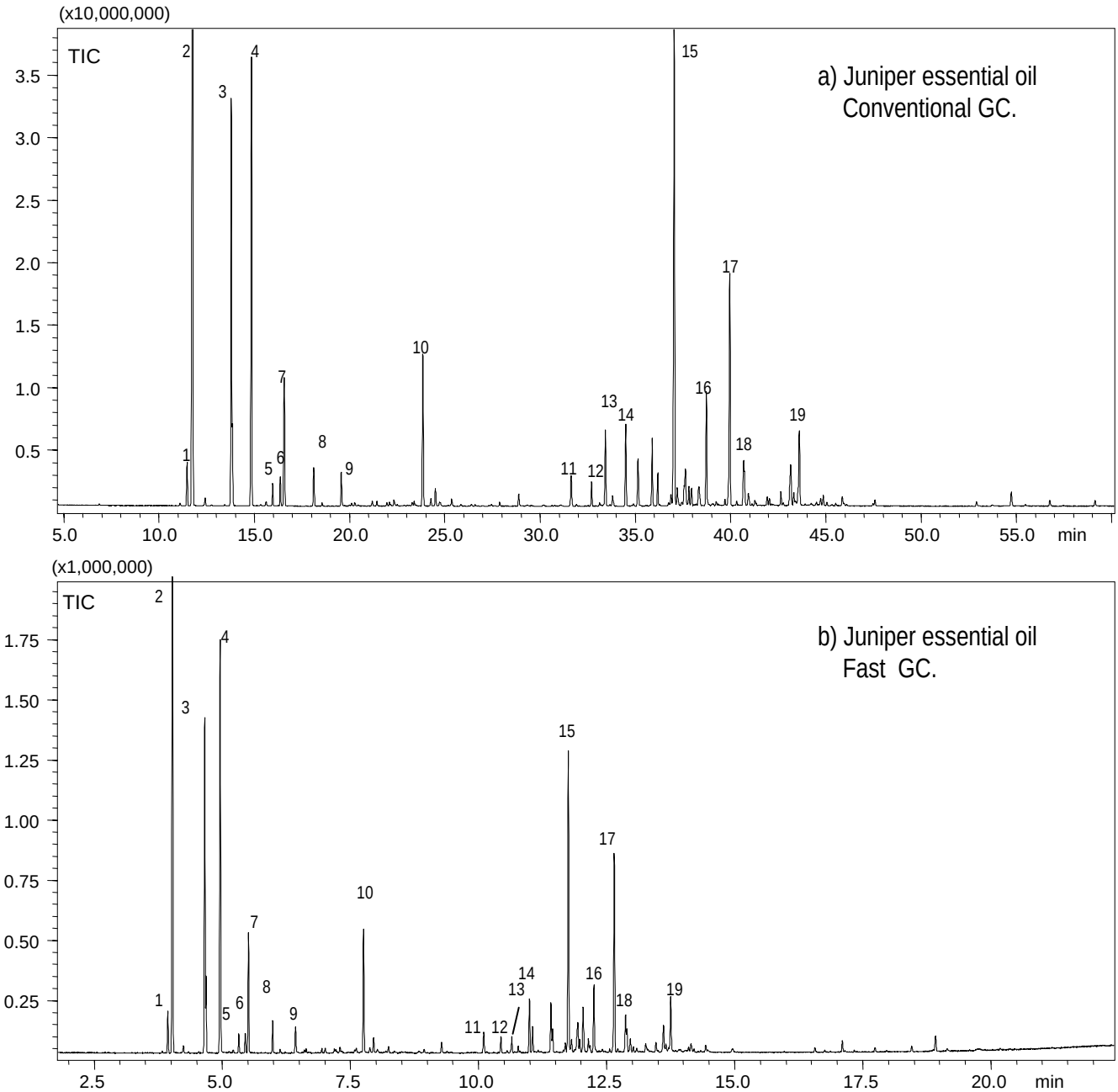
Instrumentation: Shimadzu QP2010 GC-MS system, provided with a Shimadzu AOC 5000 autosampler with SPME option, and a Shimadzu GC-MS Solution 2.51 software.

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.

a) *Juniper twigs conventional analysis*: column: 30% $6^{I\text{-}VII}$-*O*-TBDMS-$2^{I\text{-}VII}$-$3^{I\text{-}VII}$-*O*-ethyl-β-CD in PS086 25m x 0.25 mm x 0.25 µm from MEGA (Legnano - Italy). Temp. progr.: from 50°C to 220°C (2 min) at 2 °C/min. Helium in constant flow: 1mL/min.; b) *Standard marker profile*: for analysis conditions see point a; c) *Juniper twigs Fast analysis*: column dimension: 10 m x 0.10 mm x 0.10 µm. SOF analysis conditions: from 50°C to 78°C at 7.3°C/min, then to 117°C at 9.7°C/min and to 220°C at 37.4°C/min. Helium in constant flow: 0.7 mL/min.

Peak identification: 1. α-pinene, 2. β-pinene, 3. sabinene, 4 β-phellandrene., 5. limonene, 6. terpinen-4-ol. a: enantiomers (+), b: enantiomers (-).

Figure 1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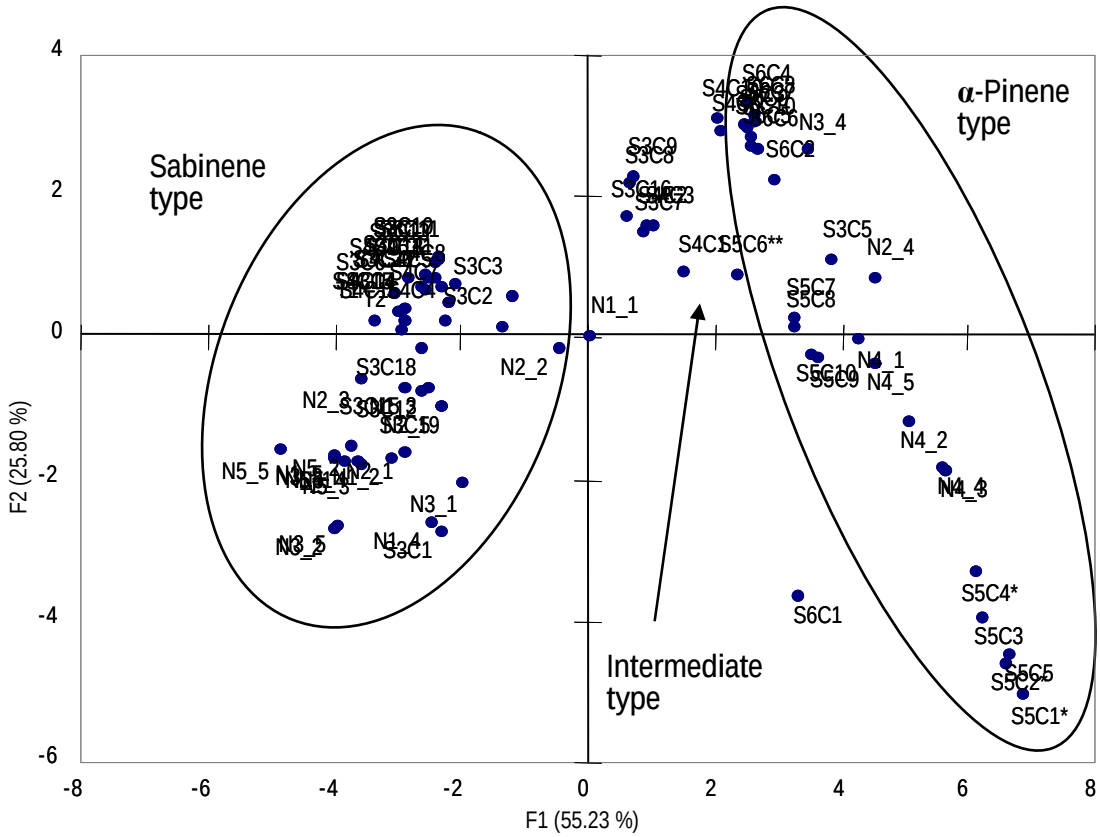
TIC
a) Juniper essential oil
Conventional GC.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
min
(x1,000,000)
TIC
b) Juniper essential oil
Fast GC.
1.75
1.50
1.25
1.00
0.75
0.50
0.25
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
min

Figure 2


Sabinene type
α-Pinene type
Intermediate type
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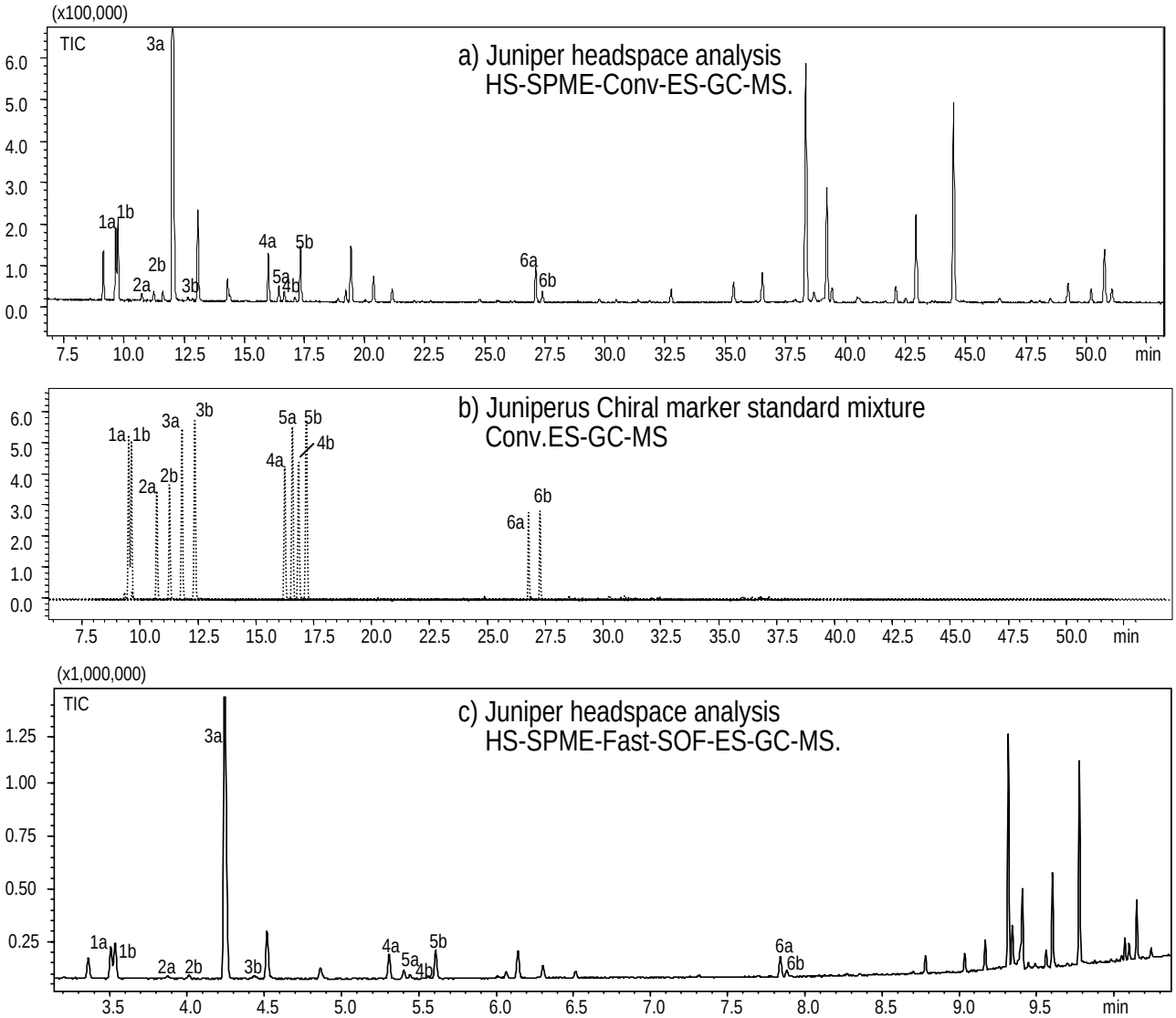
S3C2
S3C18
N2_2
N2_3
N5_5
N3_1
N1_4
S3C1
N1_1
S3C9
S3C8
S4C1
S5C6**
S6C4
N3_4
S6C2
S3C5
N2_4
S5C7
S5C8
N4_1
N4_5
N4_2
S6C1
S5C4*
S5C3
S5C5
S5C2
S5C1*
F1 (55.23 %)
F2 (25.80 %)
4
2
0
-2
-4
-6
-8
-6
-4
-2
0
2
4
6
8

Figure 3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TIC
a) Juniper headspace analysis
HS-SPME-Conv-ES-GC-MS.
1a
1b
2a
2b
3a
3b
4a
4b
5a
5b
6a
6b
min
b) Juniperus Chiral marker standard mixture
Conv.ES-GC-MS
(x1,000,000)
TIC
c) Juniper headspace analysis
HS-SPME-Fast-SOF-ES-GC-MS.