Talanta 88 (2012) 145–151

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Optimization of comprehensive two-dimensional gas-chromatography (GC × GC) mass spectrometry for the determination of essential oils

Jone Omar[a,*], Ibone Alonso[b], Maitane Olivares[a], Asier Vallejo[a], Nestor Etxebarria[a]

[a] Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country, PO Box 644, Bilbao 48080, Basque Country, Spain
[b] Idoki SFC Technologies, Technologic Park of Zamudio, Ibaizabal Bidea, Building 502, 48160 Derio, Biscay, Spain

ARTICLE INFO

Article history:
Received 28 July 2011
Received in revised form 10 October 2011
Accepted 16 October 2011
Available online 31 October 2011

Keywords:
GC × GC
Multidimensional gas chromatography
Essential oil
Experimental design

ABSTRACT

Comprehensive two-dimensional gas-chromatography (GC × GC) is a great tool to explore complex essential oils. In this work the different terpene composition of the aroma fraction of rosemary and oregano were studied. The present investigation is based on the optimization of the comprehensive two-dimensional gas-chromatographic method through the use of experimental designs and Multisimplex, and studying the modulation period, discharge-time and first and second column flows. Making use of a non-polar column (HP-5) in the first dimension and an intermediate one (DB-17) in the second dimension, we concluded that 1.42 s, 0.12 s, 1.23 mL/min and 17.55 mL/min were our optimum values, respectively. The use of a highly polar phase in the second dimension did not make any significant improvement. Finally, aroma quantification of the studied plants was performed by means of the optimum method achieved and functional groups "bands" were studied. The essential oil concentration ranges obtained were, 0.04–6.6 μg/g for rosemary extracts and 0.04–0.5 μg/g for oregano extracts.

1. Introduction

Aromatic plants synthesize and preserve a variety of biochemical products, many of which are extractable and useful as chemical feed stocks or as raw materials. Aromatic plants possess odorous volatile substances, which occur as essential oil, green exudates, balsam and oleoresin in one or more parts. The term essential oil is due to the oily aspect and to represent the essence or active constituents of plants. Essential, volatile or ethereal oils are mixtures composed by volatile liquid and solid compounds which vary widely in regard to their composition and boiling points. Plants owe their fragrance to the presence of traces of essential oils in different parts. Numerous fragrance materials are present in plants and leaves such as rosemary, lavender, or oregano, fruits like citrus and heartwoods [1].

By their nature, essential oils will range from volatile through to semi-volatile compounds. Terpenes, of which the essential oil terpenes are a sub-category, derive from the head-to-tail linkage of the isoprene $(C_5H_8)_n$ and have carbon ranges from C_{10} to C_{40} (2–8 isoprene units) [2].

Rosemary (*Rosmarinus officinalis*) is a woody, perennial herb with green needle-like leaves. It is native of the Mediterranean region [3,4]. Rosemary extracts have been reported as potent antioxidants and are a natural alternative to synthetic antioxidants [5]. Besides the therapeutic application, the essential oil is widely applied in the cosmetic industry and to preserve the shelf life of prepared food [6].

Oregano (*Labiatae*) is an annual, perennial and shrubby herb that is native of the Mediterranean, Euro-Siberian and Irano-Siberian regions. Oregano plants are widely used in agriculture and in pharmaceutical and cosmetic industries; also used as a culinary herb, flavoring substances of food products, alcoholic beverages and perfumery for their spicy fragrance [7].

The essential oils can be classified as moderately to highly complex samples comprising a wide range of classes of chemical compounds. Their complex nature dictates that, historically, very long capillary GC columns have been used to achieve adequate resolution. However, in order to identify and quantify most of the compounds we can either use chemometrical data analysis, as those pointed recently in the literature [8] or the analysis by comprehensive two-dimensional gas-chromatography (GC × GC) [3]. The possibility to separate organic complex mixtures using two capillary columns of different polarities widens remarkably the analytical strength of monodimensional GC methods, as it has already been shown in the analysis of essential oils [2,9–12].

The heart of the GC × GC coupling is the modulation. The injection into the second column is made by means of a modulator that has three main functions: accumulate and trap, refocus, and rapidly release [13]. Different modulators have been employed for GC × GC analyses. In spite of the general choice, the cryogenic modulator, the flow modulator can be an alternative [14,15]. Pulsed flow modulators grew so as the use of the GC × GC, however, Agilent

* Corresponding author. Tel.: +34 946 015 551.
E-mail address: joneomar@gmail.com (J. Omar).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.10.023

Technologies has recently introduced capillary flow technology (CFT) as a new alternative for many chromatographic systems. This modulator uses a low thermal mass deactivated stainless steel hydrogen flow device with no moving parts for switching purposes. Due to this fact, cryogen devices are not needed and pneumatics is controlled by a micro three-way solenoid valve. Within the flow modulator, analyte bands from the first column are collected in a fixed-volume channel. Then, a group of co-eluting components are successfully launched quickly into the short second column [14].

In spite of the enhanced advantages provided by GC × GC, optimization of the analytical separation is more difficult than in ordinary one dimensional gas chromatography, regardless of the detection system. Attending to the optimization requirements, multivariate and computer modeling have been widely applied in the literature in order to build easy models and templates to identify target compounds and to quantify them [16–18].

The main aim of this work is the optimization of the GC × GC set-up including a pulse flow modulator to make possible the analysis of essential oils from rosemary and oregano. In this optimization we have combined the experimental design approach to build the response surface, as it has been done in previous works [14,19], and the simplex method to explore it efficiently beyond the constrains of the experimental designs [20,21]. In addition to this, we have studied two different phases in the second dimension (an intermediate and a polar phase) in order to establish a more informative separation pattern, and we have extended this study to both mentioned essential oils.

2. Experimental work

2.1. Standard compounds

Pure standard samples of α-pinene, camphene, β-pinene, cymene, limonene, eucalyptol, γ-terpinene, linalool, camphor, borneol, α-terpineol, verbenone, bornyl acetate, eugenol, thymol and carvacrol, were supplied by Sigma–Aldrich–Fluka (Buchs, Switzerland). All of them were prepared in cyclohexane (Lab-Scan, Dublin, Ireland).

2.2. Calibration solutions

Standard stock solutions of 500 μg/g were prepared in cyclohexane and stored at 4 °C. Standard calibration solutions (1, 5, 10, 20, 30 μg/g) were prepared by weight diluting appropriate amounts of standard stock solutions in cyclohexane and stored at 4 °C too.

2.3. Samples

Three kinds of rosemary (two kinds of air-dried rosemary and freeze-dried fresh rosemary both Spanish) and an air-dried oregano were analyzed. All the plants were milled and the aromas were extracted in 10 mL cyclohexane (Lab-Scan, Dublin, Ireland) using a Focalized Ultrasound System (FUS, Bandelin, Sonopuls, HD2070 with a titanium probe MS-72) in the next conditions: 9 cycles (s^{-1}), 50% amplitude and 10 min. Finally, the samples were filtered, closed into vials and stored at 4 °C until analysis. One of the air-dried rosemary samples was steam distilled. The essential oil obtained was also injected in the GC × GC so as to see possible differences with the air-dried composition.

2.4. GC × GC analysis

GC × GC–FID/MS analyses have been performed in a GC7890A gas chromatograph (Agilent Technologies, PA, USA) equipped with a FID and 5975 C MS detector and an Agilent G-3486A valve modulator. The control of the second pressure source was handled with a pressure control module (PCM). A three-way solenoid, Fluid Automation System Valve, was used for flow switching.

The column set for GC × GC–FID/MS analysis consisted of two columns, connected by a valve modulator. Both modulation period (the time the modulator's valve is open) and discharge-time (the time the modulator needs to inject the elution of the first column into the second) have been optimized. In this study we used the following column combinations: the first dimension consisted of a HP-5MS capillary column (Agilent Technologies, 30 m × 250 μm i.d. × 0.25 μm film thickness) and the second dimension consisted of DB-17MS (Agilent Technologies, 5 m × 250 μm i.d. × 0.25 μm film thickness), whereas the other trial consisted on the same first dimension but the second dimension was an INNOWAX (Agilent Technologies, 5 m × 250 μm i.d. × 0.25 μm film thickness) column. Two deactivated but not coated fused silica tubes (restrictor) were used in order to divide the flow to FID and MS detectors, a 0.70 m, 0.32 mm id restrictor connected to FID and a 0.45 m, 0.10 mm i.d. connected to MS detector.

The operational conditions were: temperature programmed conditions from 60 to 102 °C at 4 °C/min, from 102 to 109 °C at 2 °C/min, from 109 to 161 °C at 4 °C/min, from 161 to 200 °C at 10 °C/min and from 200 to 300 °C at 20 °C/min (hold 5 min) for both columns, however, the INNOWAX temperature program ended in 250 °C and we held it for 10 min. The temperature program needed to be so convoluted as cymene, limonene and eucalyptol tended to coelute in the first dimension.

The GC was equipped with a split/splitless injector (290 °C); injections were performed in the splitless mode injecting using a 7683 Agilent autosampler. Hydrogen was employed as carrier gas (AD-1020 Hydrogen Generator, Cinel Strumenti Scientifici, Padova, Italy). Column flows were both optimized as will be shown later.

The flame ionization detector (FID) was operated at a data collection frequency of 200 Hz at 300 °C. The mass spectrometer detector (MS) worked in full scan mode from *m/z* 50 to 450, in an acquisition frequency of 12.500 amu/s (~40 scan/s) and temperatures of quadrupole and source were 150 °C and 230 °C respectively.

Data were acquired by Chemstation software (Agilent Technologies). The mono-dimensional chromatograms were processed into bi-dimensional ones by means of GC Image and GC project software (v. 2.0, Zoex Corporation, Houston, USA). This software also allowed us to create a peak corresponding matching which enables direct comparison of analyte peak responses across samples. This kind of template matching can be used to identify both targeted and non-targeted analytes in two-dimensional chromatograms in a direct way [22]. When template matching is employed for non-targeted analytes, it tries to match as many peaks as possible. As a matter of fact, this template matching has been tried with citrus essential oil analysis. Lemon, orange, mandarin and grapefruit have been examined demonstrating the template's usefulness for analyte identification.

3. Results and discussion

3.1. Optimization of the chromatographic method

The optimization of the chromatographic method was accomplished to obtain the best resolution in the separation of essential oils. This optimization includes the detailed study of key instrumental parameters by means of a Central Composite Design (CCD, The Unscrambler® v.7.5, Camo, Trondheim, Norway) and Multisimplex® (v.2, Grabitech Solutions AB, Sweden) approach. The studied parameters were: modulation period, discharge time, first column flow and second column flow. Moreover, two different second dimension columns were employed DB-17MS and INNOWAX (same physical features but, different polarity). Different

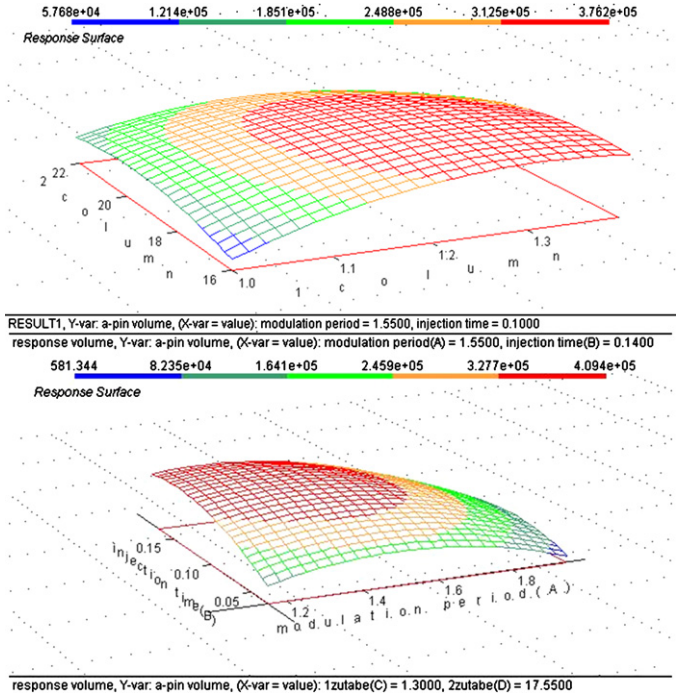

Fig. 1. Peak volume response surfaces obtained with the rosemary extracts and a DB-17MS column considering the fluxes in both columns (top) and modulation period and discharge time (bottom). The target value is the highest.

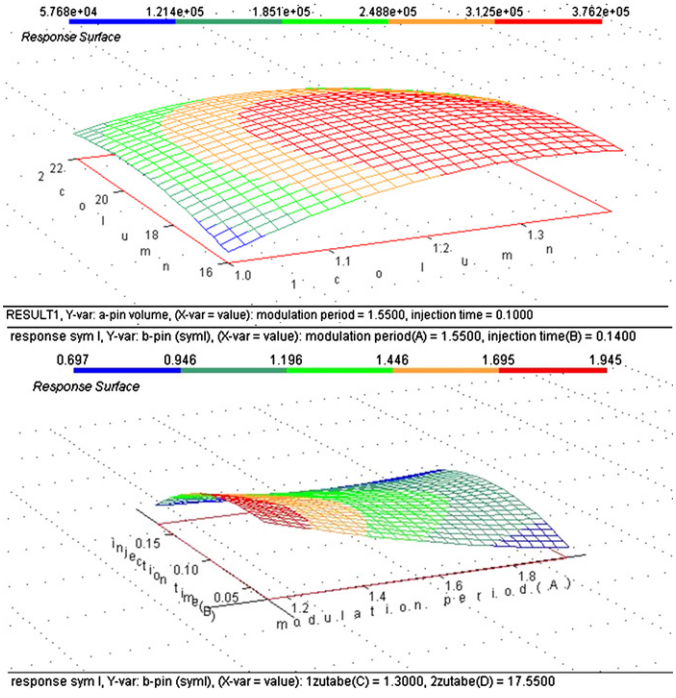

Fig. 2. Peak symmetry response surfaces obtained with the rosemary extracts and a DB-17MS column considering the fluxes in both columns (top) and modulation period and discharge time (bottom). The target value is 1.

polarities were checked in order to improve the separation in the second dimension.

Though the peak volume of each analyte can be one of the most important response variables to be considered, (since it is directly related to both the sensitivity of the analytical calibration and the resolution of nearby peaks), in GC × GC analyses other response variables can also be studied. This is the case of the symmetry of modulated peaks or the size of the peak. The former one can be estimated from the asymmetry factor (ASF) parameter (which ideally should be close to the unity). The size of the peak can be fine tune through the modulation (3–5 modulation peaks per compound) in order to get the narrowest peaks in both dimensions [23].

Several chromatographic variables can largely affect the resolution of the modulated peaks, the symmetry of the modulated peaks as well as the sensitivity of the analytical method, making necessary the right choice of the chromatographic and modulation variables.

A CCD was carried out in order to build the response surface as well as to establish the optimum working conditions for the DB-17MS column [24]. It required 27 experiments (including three replicates of the central point, and the α distance for the star points was 2) covering a factor space described in the ranges of: modulation period, 1.15–1.95 s; discharge time, 0.03–0.17 s; first column flow, 1.0–1.4 mL/min and second column flow, 16.0–22.0 mL/min. A 30 μg/g standard solution was employed for the optimization; see Table 1 for the detailed design performed (the measurements were performed in a randomized way).

The results were treated using The Unscrambler® program by both multiple linear regression (MLR) and partial least squares regression (PLS2) taking into account the peak volume and the symmetry of each identified compound [25]. Though both regression procedures (PLS2 and MLR) treat the data differently, both leaded us to the same conclusions.

Initially, considering only the peak volume, we were able to build the response surface as shown in Fig. 1a and b, concluding that the discharge time and first column flow were significant (p-level < 0.05). But considering the symmetry as can be seen in Fig. 2a and b, in addition to the discharge time and first column flow, modulation period was significant too (p-level < 0.05). The optimal values obtained in the CCD were: modulation period, 1.55 s; discharge time, 0.14 s; first column flow, 1.3 mL/min and second column flow 17.55 mL/min. As the second column flow was not significant in any of the studied cases in the CCD, it was fixed at 17.55 mL/min.

Based on those results we were interested in an integrative optimization including all the responses from all the analytes. Additionally, we were aware about the technical limitation of our microfluidic modulator, since the recommended modulation was 1.5 s when cryogenic modulators reached up to 6 s [22]. Recent literature shows that some researchers [11] make use of a flow modulation system in the GC × GC connection reaching up to 6 s in the modulation period. This technical approach was not feasible in our set-up as the equipment employed is far from the valve modulator employed in this work. Nevertheless, we wanted to evaluate the feasibility of higher modulations in our instrumental set-up and in the separation of essential oils. In this sense, the use of optimization strategies based on the Simplex method [20] allows the search of the optimum values without any previous knowledge of the response surface. Additionally, Multisimplex® allowed the simultaneous study of different responses that are included in the membership function. The membership function ranges between 0 and 1 and the closer to the overall target values of the different responses we get, this function approaches to 1 [26–28]. This way Multisimplex® made possible the integrative optimization, including the peak volume and the symmetries, and we were able to explore higher modulations cautiously.

The aim of this new optimization was once again to obtain the maximum response (considering the volume of the peak) taking into account the symmetry of each peak (close to the unity) performing the experiments one by one and checking the feasibility of the valve modulator. The step sizes of the Multisimplex® optimization were: 0.05 s for the modulation period, 0.1 s for the discharge time and 0.1 mL/min for the first column flow. The membership

Table 1
CCD design for the DB-17MS column, including the peak volume integration of the analytes.

	Parameters				Analytes response (peak volume ($\times 10^5$))								
	Mod. period (s)	Injection time (s)	1st col. (mL/min)	2nd col. (mL/min)	α-Pinene	Camphene	β-Pinene	Cymene	Limonene	Eucalyptol	Camphor	Borneol	α-Terpineol
1	1.15	0.10	1.2	19	2.4	3.3	1.6	2.8	3.6	39.4	11.0	2.4	2.7
2	1.95	0.10	1.2	19	2.5	4.4	1.6	4.3	5.1	46.5	12.3	3.2	3.7
3	1.55	0.03	1.2	19	2.3	3.2	1.0	2.6	3.6	28.0	8.0	2.4	2.4
4	1.55	0.17	1.2	19	3.3	2.6	1.4	3.2	4.4	33.4	9.0	2.8	3.6
5	1.55	0.10	1	19	2.3	3.4	2.0	3.4	4.9	34.2	11.5	2.5	2.9
6	1.55	0.10	1.4	19	3.0	4.8	2.0	3.9	6.8	50.1	14.4	4.1	4.2
7	1.55	0.10	1.2	16	3.3	5.0	1.4	4.0	6.6	45.6	12.1	3.7	4.4
8	1.55	0.10	1.2	22	2.7	3.7	1.8	3.9	6.1	40.5	11.7	3.3	3.3
9	1.35	0.06	1.1	17.5	1.7	2.6	1.3	2.5	3.2	25.7	6.8	2.5	2.6
10	1.75	0.06	1.1	17.5	1.3	2.3	1.1	1.8	3.5	18.0	6.1	1.6	2.0
11	1.35	0.14	1.1	17.5	2.4	4.5	1.6	4.7	5.3	47.3	13.3	3.8	3.7
12	1.75	0.14	1.1	17.5	2.4	4.3	1.5	3.6	5.8	45.5	11.7	3.5	3.9
13	1.35	0.06	1.3	17.5	3.6	1.8	1.7	3.0	2.7	18.9	5.0	2.4	2.0
14	1.75	0.06	1.3	17.5	2.4	3.4	1.4	2.7	5.4	33.7	9.0	2.6	2.9
15	1.35	0.14	1.3	17.5	3.8	4.7	1.9	5.4	6.7	53.5	15.2	3.4	4.7
16	1.75	0.14	1.3	17.5	2.5	3.5	1.9	3.9	5.4	53.0	12.4	3.6	3.5
17	1.35	0.06	1.1	20.5	1.5	2.3	1.2	3.0	3.4	23.9	7.5	2.2	2.0
18	1.75	0.06	1.1	20.5	2.5	2.4	0.9	1.8	3.1	18.0	4.5	1.5	1.9
19	1.35	0.14	1.1	20.5	2.5	3.4	1.5	3.8	4.6	36.3	10.3	2.7	3.6
20	1.75	0.14	1.1	20.5	1.8	2.1	1.7	3.5	4.8	32.2	9.6	2.1	2.7
21	1.35	0.06	1.3	20.5	2.3	3.6	1.7	1.7	3.1	16.5	4.7	1.9	2.1
22	1.75	0.06	1.3	20.5	2.0	2.4	1.3	2.4	4.4	22.9	6.7	1.9	2.0
23	1.35	0.14	1.3	20.5	3.7	4.7	1.8	4.7	6.8	48.2	15.1	4.1	3.8
24	1.75	0.14	1.3	20.5	2.1	3.5	1.5	3.3	4.8	38.3	12.2	2.8	3.4
25	1.55	0.10	1.2	19	3.9	4.2	2.0	4.5	3.9	48.0	13.6	3.6	3.9
26	1.55	0.10	1.2	19	3.7	4.2	1.4	3.7	4.6	38.5	11.0	3.2	3.2
27	1.55	0.10	1.2	19	3.2	3.6	1.5	4.0	5.2	47.0	12.6	3.8	4.0

Table 2
Rosemary aroma concentration in μg/g of dried plant (nd stands for non-detected).

Aroma	Dried rosemary 1		Dried rosemary 2		Freeze-dried rosemary		Dried oregano	
	μg/g	RSD%	μg/g	RSD%	μg/g	RSD%	μg/g	RSD%
α-Pinene	1.9	7	1.3	6	1.9	4	nd	
Camphene	0.5	4	0.7	3	0.9	3	nd	
β-Pinene	0.3	7	0.1	6	0.3	3	nd	
p-Cymene	0.2	5	0.2	6	0.1	8	0.4	2
Limonene	0.4	2	0.4	2	0.5	5	nd	
Eucalyptol	6.6	4	3.1	4	2.7	2	nd	
γ-Terpinene	0.04	3	nd		0.1	9	0.3	9
Linalool	0.2	4	nd		0.03	2	0.3	3
Camphor	3	4	3.3	3	2.8	3	nd	
Borneol	0.7	3	0.9	3	0.5	4	nd	
α-terpineol	0.7	4	0.5	4	0.4	2	0.04	4
Verbenone	nd		nd		1.2	4	nd	
Bornyl acetate	nd		nd		0.8	3	nd	
Thymol	0.07	5	nd		nd		0.4	2
Karvakrol	0.03	2	nd		nd		0.5	1

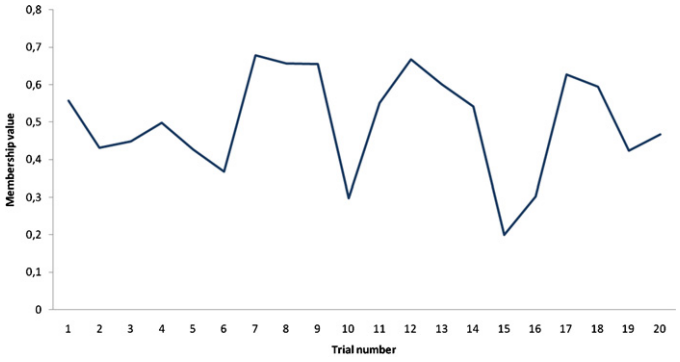

Fig. 3. Membership values of the Multisimplex trials.

function allows us to select the relative significance of the responses being optimized, as well as how the significance of each response being optimized changes as the optimization progresses. Finally, the responses did not take us very far. After 20 experiments, in the last simplex, the parameter value ranges were: modulation period, 1.35–2.3 s; discharge time, 0.054–1.37 s and 1st column flow, 1.19–1.39 mL/min. The highest membership was obtained in trials 7, 8 and 12, so an intermediate optimal value was chosen, and therefore the optimum values were fixed at: 1.42 s for the modulation period, 0.12 s for the discharge time and 1.23 mL/min for the first column flow (the second column flow was already fixed at 17.55 mL/min). All the trials' membership is illustrated in Fig. 3.

We changed the second column (Innowax instead of DB-17MS) in order to achieve a better orthogonality. For this purpose a new Multisimplex design was carried out based on the optimum values of the previous CCD. The same step size (0.05 s for the modulation period, 0.1 s for the discharge time and 0.1 mL/min for the first column flow) as with the previous column was employed to perform the design. It is worth mentioning that the spot size and the symmetry in both dimensions were enhanced with the polar column. Optimum values obtained for the studied parameters with the Innowax column were 1.32 s for the modulation period; 0.038 s for the discharge time and 1.15 mL/min for the 1st column flow respectively. However, as it will be explained later on, DB-17 was chosen to perform the analyses of this work.

3.2. Real samples quantification

Once the optimum chromatographic method was achieved, real samples quantification was performed with the DB-17MS column in the second dimension. In the case of rosemary, three different type of samples were analyzed, rosemary of two origins but air-dried, and one freeze-dried. In the case of oregano only the air-dried plant was analyzed.

The aroma extraction can be a key step when referring to essential oil analysis as it has been previously studied [29], the conditions employed in this work for both rosemary and oregano were studied beforehand. The aroma extracts were obtained by means of focused ultrasound extraction, and the process was fully optimized following the procedure described in the literature [22]. In this case, we used 0.25 g of dried plant that were extracted with 10 mL of cyclohexane with a titanium probe. The sonication time was 10 min at 50% of amplitude and 9 cycles (s^{-1}).

One of the main drawbacks of our GC × GC–FID/MS set-up is the low sensitivity, essentially because the flow modulator is well-suited to flame ionization detectors, where the high fluxes are not a drawback. When a MS detector is required the eluting flow of the second column should be split in two with a flow ratio of 1:10 (MS:FID) to assure a good performance of the MS detector.

A calibration curve was built from 1 to 30 μg/g and, as in the case of the optimization step; the volume of the peak was taken as response. Good determination coefficients were obtained ($r^2 > 0.965$) for all the analytes and the repeatability of the chromatographic method as the RSDs % were within 1–9% for all the analytes. The detection limits were calculated from the calibration parameters using the offset plus three times its standard deviation. The detection limits calculated in this way ranged between 1 and 6 μg/g. When the FID responses were considered instead of the MS ones, roughly 10 times lower values were obtained. However, when comparing both chromatographic data acquisition, MS allows the above mentioned template matching (target analytes in calibrations), automatic integration and mismatching avoidance. That is why only MS results are discussed in this work.

In general terms, as shown in Table 2, no statistical differences exist between both air-dried rosemary kinds. However, the freeze-dried rosemary shows less aroma concentration than the air dried ones. Some of the studied aromas have been detected in rosemary and oregano. As can be appreciated the most abundant analyte is eucalyptol, in order to pre-concentrate the rest of the analytes a nitrogen blow-down evaporation at the Turbovap LV Evaporator was employed and the extract was prepared in 1.5 mL of cyclohexane. However, due to the volatility of the compounds, the first eight eluting monoterpenes were completely lost in the nitrogen stream (until t_R 15 min). Spiking rosemary with oregano standard compounds and vice versa was tried in order to quantify these losses. Depending on the volatility of the compound,

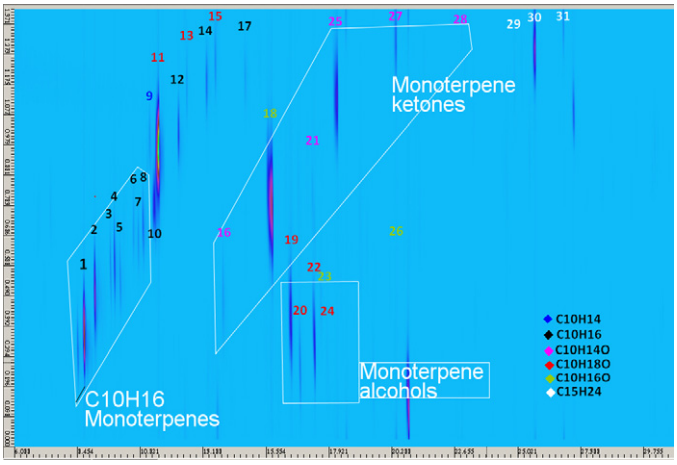

Fig. 4. Rosemary aromas separated with a DB-17MS in the 2nd dimension. The type of terpernes is highlighted and the molecular structure of the eluting compounds is indicated with a different color.

90% was lost (t_R < 15 min) and 20% was lost for less volatile ones (t_R > 15 min).

In addition to this, we wanted to compare the steam-distilled and FUS extracted rosemary extracts. Based on a correlation analysis among all the extracts (steam-distilled, freeze-dried and air-dried rosemary) all of them showed high correlation indexes ($0.81 < r^2 < 0.99$) but freeze-dried extract showed the lowest values. This would suggest that the aromatic features are kept regardless the pretreatment of the sample, and that loses may happen during freeze-drying process. Moreover, the optimized chromatographic method can be employed for the analysis of rosemary essential oils obtained by means of different extraction techniques.

3.3. Real samples compound identification

Contour plot peak identification was achieved through information derived from pure standards and NIST08 mass-spectra library. Depending on the molecular composition of the aroma compounds they tend to elute within specific zones in the bidimensional chromatogram. The ability to separate compounds based on chemical class is characteristic of GC × GC. The ability to structure the chromatogram into chemical compound "bands" is important, because, it is now possible, without mass spectral identification, to qualitatively assign the nature of the chemical class of the unidentified compound [9].

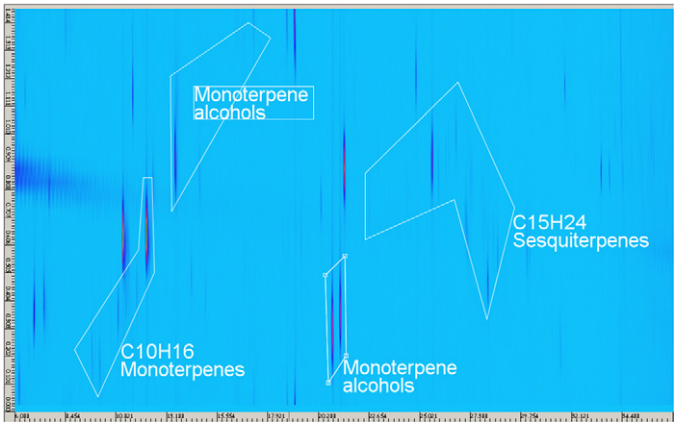

Fig. 5. Oregano terpenes separated with a DB-17MS in the 2nd dimension, terpenes divided by functional families.

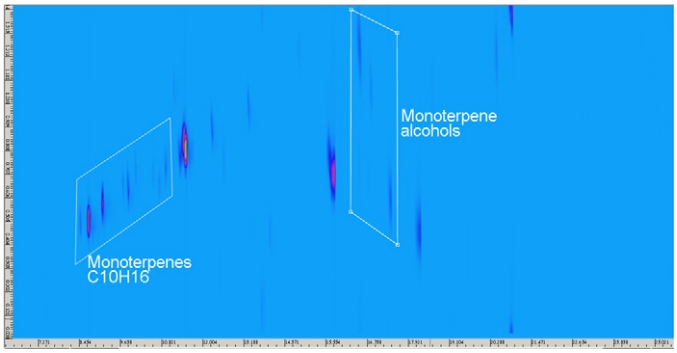

Fig. 6. Rosemary terpenes functional group separation with an Innowax column in the 2nd dimension.

All the quantified compounds were monoterpenes, however, the qualitatively studied aromas were monoterpenes and sesquiterpenes. Considering the isoprene structure of most of essential oils, the most frequent observed structures were: $C_{10}H_{16}$, $C_{10}H_{14}O$, $C_{10}H_{16}O$, $C_{10}H_{18}O$, $C_{15}H_{24}$. We did not go very far in the sesquiterpene study because most of the detected compounds were isomers, they eluted late and the separation requirements made it difficult to separate and identify.

In Fig. 4 we identified most common aromas of the rosemary by colors, referring to each molecular composition (this way different clusters can be observed). In the next two figures, Figs. 5 and 6, a clustering according to functional groups can be observed in the rosemary and oregano analyses. When we compare second column combinations, DB-17MS showed a better clustering of monoterpenes with the same functional groups, and in addition to this, the sensitivity of the DB-17MS column was slightly better. In Fig. 6 it is shown the chromatogram of a rosemary extract obtained with the Innowax column. The chromatographic conditions and the samples are the same in both cases (Figs. 4 (DB17-MS) and 6 (Innowax)), but in the later case less compounds are observed, the most abundant monoterpenes got distorted, and the functional grouping was not so straightforward.

Table 3
Identified terpenes in rosemary plant extract.

Peak	R_t (min)	Name (common)	Molecular formula
1	8.722	1R-α-Pinene	$C_{10}H_{16}$
2	9.126	Camphene	$C_{10}H_{16}$
3	9.696	β-Thujene*	$C_{10}H_{16}$
4	9.861	β-Pinene	$C_{10}H_{16}$
5	10.073	β-Myrcene	$C_{10}H_{16}$
6	10.573	α-Phellandrene*	$C_{10}H_{16}$
7	10.762	β-Ocimene*	$C_{10}H_{16}$
8	10.928	2-Carene*	$C_{10}H_{16}$
9	11.170	p-cymene	$C_{10}H_{14}$
10	11.309	Limonene	$C_{10}H_{16}$
11	11.431	Eucalyptol	$C_{10}H_{18}O$
12	12.283	γ-Terpinene	$C_{10}H_{16}$
13	12.593	unknown	$C_{10}H_{18}O$
14	13.327	Terpinolene*	$C_{10}H_{16}$
15	13.660	β-Linalool	$C_{10}H_{18}O$
16	13.931	Chrysanthenone*	$C_{10}H_{14}O$
17	14.771	unknown	$C_{10}H_{16}$
18	15.712	Camphor	$C_{10}H_{16}O$
19	16.485	Borneol	$C_{10}H_{18}O$
20	16.838	4-Terpineol*	$C_{10}H_{18}O$
21	17.108	p-Cymen-8-ol*	$C_{10}H_{14}O$
22	17.384	α-Terpineol	$C_{10}H_{18}O$
23	17.623	Myrtenol*	$C_{10}H_{16}O$
24	17.856	Santolina alcohol*	$C_{10}H_{18}O$
25	18.248	Verbenone	$C_{10}H_{14}O$
26	20.204	β-Citral	$C_{10}H_{16}O$

* Means Exact isomer not determined, name predicted by NIST 08.

Table 4
Identified terpenes in oregano plant extract.

Peak	R_t (min)	Name (common)	Molecular formula
1	8.460	α-Thujene[a]	$C_{10}H_{16}$
2	9.695	Sabinene[a]	$C_{10}H_{16}$
3	10.073	β-Myrcene	$C_{10}H_{16}$
4	10.928	Terpinolene[a]	$C_{10}H_{16}$
5	11.169	β-Cymene[a]	$C_{10}H_{14}$
6	11.308	β-Terpinyl acetate[a]	$C_{12}H_{20}O_2$
7	11.805	3-Carene[a]	$C_{10}H_{16}$
8	12.259	γ-Terpinene	$C_{10}H_{16}$
9	12.568	Unknown	$C_{10}H_{18}O$
10	13.635	Linalool	$C_{10}H_{18}O$
11	13.706	Nerolidol[a]	$C_{15}H_{26}O$
12	14.747	Z-neo-allo-ocimene[a]	$C_{10}H_{16}$
13	16.484	Borneol	$C_{10}H_{18}O$
14	17.289	α-Terpineol	$C_{10}H_{18}O$
15	19.228	Thymol methyl ether[a]	$C_{11}H_{16}O$
16	19.524	Thymoquinone[a]	$C_{10}H_{12}O_2$
17	21.032	Thymol	$C_{10}H_{14}O$
18	21.412	Karvakrol	$C_{10}H_{14}O$
19	22.697	Elixene[a]	$C_{15}H_{24}$
20	24.093	α-Cubebene	$C_{15}H_{24}$
21	24.426	β-Bourbonene[a]	$C_{15}H_{24}$
22	25.659	β-Caryophyllene[a]	$C_{15}H_{24}$

[a] Exact isomer not determined, name predicted by NIST08.

Considering all the isomers present in these extracts, individual identification can be cumbersome. It has to be mentioned that as the flow is divided between the MS and FID in a 1:10 ratio, much more compounds can be detected in the FID. However, once again the NIST08 library cannot be applied and without standard compounds their identification is not possible. We have been able to identify up to 30 compounds for both rosemary and oregano in the MS, however, a considerable number of unidentified compounds are still left. Regarding monoterpenes many $C_{10}H_{16}$ isomers have been found in the first eluting part and many $C_{15}H_{24}$ isomers in the less volatile part. Functional group clustering helps assigning unknown compounds by their location, the NIST08 mass-spectra library helps identifying the molecular composition of the analytes but, certain isomer identification seems to be cumbersome. All the identified terpenes in rosemary and oregano are shown in Tables 3 and 4 respectively.

4. Conclusions

As we have seen up to now, plants extracts are very complex mixtures. Just having studied only the terpenes, we have made out how rich can it be, and, above all, the importance of a good strategy to develop a GC × GC separation. In this sense, we have shown the combination of both experimental designs and simplex based algorithms to a GC × GC parameter optimization. Based on this development we were able to identify many terpenes and to quantify most of them. In addition to this, since we have combined two different phases in the second dimension, we were able to improve the identification of unknown complex mixtures based on the clustering patterns observed.

Finally, GC × GC–MS/FID is not the best approach to a quantitative issue, especially when we deal with traces as a consequence of the low detection limits of our instrumental set-up, but when the target analytes are clearly above the detection limits, this option can be the simplest one because we simplify remarkably the burden of the GC separation.

Acknowledgements

Jone Omar and Ibone Alonso are grateful to the Basque Government for their PhD fellowship and to IDOKI SCF Technologies S.L. Moreover, this work has been financially supported by DIPE 09/09 project of the Council of Biscay.

References

[1] S. Clarke, Essential Chemistry for Aromatherapy, Churchill Livingstone, UK, 2009.
[2] P. Marriott, R. Shellie, C. Cornwell, J. Chromatogr. A 936 (2001) 1–22.
[3] M. Adahchour, J. Beens, R.J.J. Vreuls, U.A.Th. Brinkman, Trends Anal. Chem. 25 (2006) 821–840.
[4] M. Adahchour, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A 1186 (2008) 67–108.
[5] F.J. Señoráns, E. Ibañez, S. Cavero, J. Tabera, G. Reglero, J. Chromatogr. A 870 (2000) 491–499.
[6] N. Bousbia, M. Vian, M. Ferhat, E. Petitcolas, B. Meklati, F. Chemat, Food Chem. 114 (2009) 355–362.
[7] B. Bayramoglu, S. Sahin, G. Sumnu, J. Food. Eng. 88 (2008) 535–540.
[8] M. Jalali-Heravi, R.S. Moazeni, H. Sereshti, J. Chromatogr. A 1218 (2011) 2569–2576.
[9] M.M. Perera-Ranjini, P.J. Marriott, I.E. Galbally, Analyst 127 (2002) 1601–1607.
[10] J. Dimandja, S. Stanfill, J. Grainger, D. Patterson, J. High Resolut. Chromatogr. 23 (2000) 208–214.
[11] L.A. Fonseca de Godoy, L.W. Hantao, M.P. Pedroso, R.J. Poppi, F. Augusto, Anal. Chim. Acta 699 (2011) 120–125.
[12] D. Sciarrone, R. Costa, C. Ragonese, P.Q. Tranchida, L. Tedone, L. Santi, P. Dugo, G. Dugo, D. Joulain, L. Mondello, J. Chromatogr. A 1218 (2011) 137–142.
[13] H. Cortes, B. Winniford, J. Luong, M. Pursch, J. Chromatogr. A 32 (2009) 883–904.
[14] M. Olivares, M. Irazola, A. Vallejo, X. Murelaga, O. Zuloaga, N. Etxebarria, J. Chromatogr. A 1218 (2011) 1656–1662.
[15] A. Vallejo, M. Olivares, L.A. Fernández, N. Etxebarria, S. Arrasate, E. Anakabe, A. Usobiaga, O. Zuloaga, J. Chromatogr. A 1218 (2011) 3064–3069.
[16] F.L. Dorman, P.D. Schettler, L.A. Vogt, J.W. Cochran, J. Chromatogr. A 1186 (2008) 196–201.
[17] C. Cordero, E. Liberto, C. Bicchi, P. Rubiolo, P. Schieberle, S. Reichenbach, Q. Tao, J. Chromatogr. A 1217 (2010) 5848–5858.
[18] S. Reichenbach, P. Carr, D. Stoll, Q. Tao, J. Chromatogr. A 1216 (2009) 3458–3466.
[19] A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Anal. Bioanal. Chem. 390 (2008) 739–748.
[20] J.A. Nelder, R. Mead, Comput. J. 7 (1965) 308–313.
[21] O. Zuloaga, N. Etxebarria, L.A. Fernández, J.M. Madariaga, J. Chromatogr. A 849 (1999) 225–234.
[22] P. Navarro, N. Etxebarria, G. Arana, Anal. Chim. Acta 648 (2009) 178–182.
[23] J. Dallüge, J. Beens, U.A.Th. Brinkman, J. Chromatogr. A 1000 (2003) 69–108.
[24] R. Leardi, Anal. Chim. Acta 652 (2009) 161–172.
[25] K.H. Esbensen, Multivariate Data Analysis – In Practice, 5th ed., CAMO, 2001.
[26] N. Ferreirós, G. Iriarte, R.M. Alonso, R.M. Jiménez, Talanta 69 (2006) 747–756.
[27] O. Zuloaga, N. Etxebarria, L.A. Fernández, J.M. Madariaga, Anal. Chim. Acta 416 (2000) 43–53.
[28] E. Cortazar, O. Zuloaga, J. Sanz, J.C. Raposo, N. Etxebarria, L.A. Fernández, J. Chromatogr. A 978 (2002) 165–175.
[29] M. Jalali-Heravi, H. Parastar, H. Ebrahimi-Najafabadi, J. Chromatogr. A 1216 (2009) 6088–6097.