Routine quantification of 54 allergens in fragrances using comprehensive two-dimensional gas chromatography-quadrupole mass spectrometry with dual parallel secondary columns. Part I: Method development

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Emilie Belhassen[a], Davide Bressanello[b], Philippe Merle[a], Elsa Raynaud[a], Carlo Bicchi[b], Alain Chaintreau*[a], Chiara Cordero*[b]

[a]*Firmenich SA, Corporate R&D Division, Route des Jeunes 1, CH-1211 Geneva 8*

[b]*Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via P. Giuria 9, I-10125 Torino, Italy*

* Corresponding authors:

- Chiara Cordero, Dipartimento di Scienza e Tecnologia del Farmaco, Università degli studi Torino, Via Pietro Giuria 9, I-10125 Torino, Italy. E-mail: chiara.cordero@unito.it; Phone: +39 0116707662; Fax: +39 0112367662
- A. Chaintreau, Firmenich SA, Corporate R&D Division, Route des Jeunes 1, CH-1211 Geneva 8, Switzerland. E-mail: alc.ffj@sfr.fr; Phone: + 41 22 780 34 71; Fax: + 41 22 780 33 34

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1 Abstract

The forthcoming extension of the regulated fragrance allergen number is raising a challenge: the previously developed quantification methods need to be drastically improved in terms of rapidity, without altering their reliability. This work describes a procedure based on comprehensive bidimensional gas chromatography, hyphenated with a low-cost quadrupole mass spectrometer (GCxGC-QMS). The second chromatographic dimension was operated in a dual configuration, consisting of two parallel secondary columns, one being connected to the QMS, the other to a flame ionization detector (FID). This not only allowed optimization of the

chromatographic gas flows when compared to the usual single second dimension, but it also enabled the use of the FID signal to extend the quantification range from 2 to 10,000 mg/kg. The QMS was used at a low level (2-100 mg/kg), where coelutions are frequent, and the FID was used for the most abundant analytes (100-10,000 mg/kg) with a low risk of significant interferences. To assess the identity and purity of the analyte peak at low level, we propose a strategy and summarize it in a decisional tree. To our knowledge, this represents the first fully developped quantification method based on a GCx2GC-QMS/FID system. In contrast to other methods, it enables the quantification of a wide variety of analytes in fragrances in a single run, without requiring a series of sample dilutions to match the calibration range.

2 Introduction

2.1 Regulation and related quantification methods

2.1.1 First regulation and analysis of 24 volatile allergens

In 1999, the Scientific Committee on Cosmetic Products and Non-Food Products published a list of 24 compounds identified as "the most frequently recognised allergens."[(1)] Four years later, a European Directive regulated these 24 allergens,[(2)] and the International Fragrance Association (IFRA, www.ifraorg.org) published a GC-MS method for their quantification in the range of 10-100 mg/L,[(3)] corresponding to the no-declaration limits for leave-on and rinse-off products, respectively. This method represented the first case of multianalyte quantification in the domain of flavors and fragrances. Each compound was monitored by using three ions, the abundance ratios of which also being used to confirm analyte identities.[(4)] The analysis was duplicated on two different column stationary phases to overcome possible coelutions. The method was submitted to a circular test at the Centre Européen de Normalisation and validated[(5)] before being adopted as a European Norm.[(6)] Many GC-MS methods derive from the initial IFRA procedure, either by selected ion monitoring (SIM),[(7, 8)] or by extracting these ions from a scan acquisition.[(9-11)]

2.1.2 Revision of the allergen list and possible analytical methodologies

In an opinion published in 2011, the Scientific Committee on Consumer Safety proposed to the European Commission a new list of "established contact allergens in humans" (Table 13-1),[(12)] consisting of 54 chemicals and 28 natural extracts. Because several of these chemicals exist as different isomeric structures, monitoring them in fragrances led to the quantification of 67 analytes. The application of the same approach as the European Norm to the new allergen list would lead to too many analytes in a single analysis and a major risk of coelution. Alternatively,

the analytes could be divided into two sets and analyzed in two separate GC-MS runs. This would be significantly time-consuming because it would imply four injections for each single sample (2 columns x 2 sets of analytes). Performing this quantification in a single run requires drastic enhancement of the performances of the analytical system, either by improving the GC separation or by increasing the detection specificity.

In previous work, we calculated the occurrence probability of two compounds giving rise to a coelution on both column phase polarities and exhibiting the same three monitored ions.[4] This probability was significant and confirmed by experimental observations. To overcome this difficulty, one can increase the detection selectivity by using tandem and/or high-resolution mass spectrometry. However, both coeluted compounds may have the same molecular formula: for instance, monoterpene hydrocarbons, alcohols or ketones are frequent constituents of fragrances. Such compounds with identical molecular formulae often lead to the same excited species after electron impact ionization, so that the parents have the same exact mass, as well as the same product ions. This risk suggests that a higher MS selectivity would not drastically improve the performance of a quantification method of fragrance allergens.

The second alternative, i.e. improving GC separation, can be achieved in two ways. The first is based on multidimensional GC, using two different column polarities: the chromatogram of the first column is divided into zones around the allergens to be quantified, and each zone is transferred to the second column connected to the MS.[13] This approach has recently been extended to the new list of 54 chemically defined allergens,[12] but it requires two different injections to allow insertion of 14 to 17 cut segments in each chromatographic run, which increases the time spent per sample.[14] The second way involves comprehensive bidimensional GC (GCxGC). A first attempt at using a flame ionization detector (GCxGC-FID) suffered from the same limits as GC-MS because both techniques are bidimensional and do not solve coelution issues.[15] Therefore, a third dimension, such as MS detection, should be considered. The hyphenation of time-of-flight MS (TOFMS) has given rise to many quantitative applications.[16] But the cost of such an instrument remains a limitation in the context of a quality control laboratory; to the best of the authors' knowledge, no fully developed GCxGC-TOFMS quantification method for allergens has been published to date on this topic. The first hyphenation of GCxGC with a low-cost quadrupole MS (QMS) was made in 2004 and tested on allergens in SIM mode.[17] If satisfactory results were obtained, the analyte monitoring was based on only a single ion because of the low sampling rate of QMS at the time (30.7 Hz). As a consequence, the analyte identity could not be checked. The capabilities of rapid-scanning QMS were tested on allergens, without developing a quantification method.[18, 19] The first validated GCxGC-QMS method dedicated to the quantification of allergens was published in 2007: good

linearities, precisions and accuracies were observed.[20] Recently, the capabilities of a GCxGC-QMS based on a flow modulator were tested with the new allergen list.[21] Large peak widths of 600 ms were observed, which impairs the resolution of adjacent peaks in the second dimension. The performances were also not evaluated by spiking the allergens in a representative matrix.

Except for the aforementioned papers on allergens,[17, 20] only a few publications have reported the development of a full multianalyte quantification using GCxGC-QMS.[22-27] In most of them, the method was tested by using a matrix spiked with known concentrations of analytes.

The typical design of a comprehensive GCxGC system consists of an online connection of a conventional capillary column (typically 0.25 mm I.D.) to a fast GC column (typically 0.1 mm I.D.). The resulting drawback lies in the compromise in terms of carrier gas velocity because the diameter difference between these dimensions does not allow an optimal velocity to be reached in both columns (usually 150 cm/s in the second dimension instead of 20-35 cm/s). The problem was first addressed by Tranchida et al.,[28] who proposed adding an adjustable split at the interface between both columns. Similarly, Gu et al.[29] and then Peroni et al.[30] and Nicolotti et al.[31] connected two fast GC columns after the first dimension to enable the simultaneous use of two detectors and decrease the overpressure at the interface. This alternative will be tested in the present work. Alternatively, Klee et al propose the use of two columns of identical diameter (e.g., 0.25 mm I.D.)under optimized flow conditions. However this configuration generates average peak-widths of 20 ms at half-height, that are incompatible with the scan rate of a quadrupole [32].

The use of dedicated software is crucial for quantitative GCxGC because of the huge amount of raw data it generates. LECO has developed proprietary software called ChromaTOF® that enables quantitation workflows using GCxGC-TOFMS data.[33] Several quantitative applications have been published (e.g.[34, 35]), but this software is exclusively dedicated to data treatment of files acquired by LECO's instruments. The University of Messina has developed a software platform supporting the elaboration of LC×LC and GC×GC data called ChromSquare® (Chromaleont, Messina, Italy), distributed by Shimadzu.[36] This software has a dedicated function for quantitative analysis and is compatible with other manufacturers' input files.[21] GC Image®, which was introduced in 2001 and developed at the University of Nebraska,[37] is an attractive solution because it also provides useful tools for pattern recognition. It enables data processing from single-channel (FID, FPD, SSD) and spectral detectors (MS, diode array, etc). It is also compatible with raw data from various sources. GC Image has often been used either for quantitative profiling of complex mixtures (e.g.[38, 39]) or for quantification of targeted analytes by internal standardization (e.g.[22, 24]). This software was chosen for this study because of its versatility and performance.

The objective of this work is to provide quality control laboratories with a routine, cost-effective GCxGC-MS technique to quantify all 54 suspected allergens and their isomers in a single run. A low-cost QMS was therefore selected as a detector. This work also aims to determine the optimal GCxGC separation and QMS conditions and to investigate the capabilities of GC Image software for the specific needs of such multianalyte quantification.

3 Experimental

3.1 Chemicals

All standards used for the development of the GCxGC-QMS method came from Sigma-Aldrich (Steinheim, Germany), as did the internal standards (ISTDs), 1,4-dibromobenzene and 4,4'-dibromobiphenyl, and the solvents, methyl pivalate and methy tert-butyl ether (MTBE) (Table 1). The fragrance model ("Lili"), composed of 39 constituents, was free of all allergens (Table 1).

Table 1. Allergen standards and fragments Q1, Q2 and Q3 used for their MS quantification

[a]	N°	Compound	CAS #	Purity [b]	Q1	Q2	Q3
A	1	Amylcinnamic alcohol alpha	101-85-9	>99%	133	91	204
A	2	Anethole trans	4180-23-8	>99%	148	147	117
A	3	Anise alcohol	105-13-5	>99%	138	109	121
A	4	Benzyl alcohol	100-51-6	100%	79	108	107
A	5	Caryophyllene beta	87-44-5	99%	91	133	204
A	6	Cinnamyl alcohol	104-54-1	98%	92	134	115
A	7	Citronellol	106-22-9	>99%	69	41	156
A	8	Ebanol® E	1067999-31-8	45%	149	83	93
A	9	Ebanol® Z	1237530-53-8	45%	149	69	55
A	10	Eugenol	97-53-0	100%	164	149	131
A	11	Farnesol (E, E)	106-28-5	99%	69	81	93
A	12	Geraniol	106-24-1	99%	69	138	123
A	13	Isoeugenol E	5932-68-3	>99%	164	149	103
A	14	Isoeugenol Z [e]	5912-86-7	<1%	164	149	103
A	15	Limonene	138-86-3	>99%	68	67	136
A	16	Linalool	78-70-6	>99%	71	93	121
A	17	Menthol	89-78-1	>99%	81	71	95
A	18	Pinene alpha	80-56-8	>99%	93	91	136

A	19	Pinene beta	127-91-3	99%	93	79	69
A	20	Santalol alpha	115-71-9	52%	93	202	107
A	21	Santalol beta	77-42-9	23%	94	122	79
A	22	Sclareol	515-03-7	99%	69	191	177
A	23	Terpinene alpha	99-86-5	90%	121	93	136
A	24	Terpineol alpha	98-55-5	92%	136	121	93
A	25	Trimethylbenzene propanol (Majantol®)	103694-68-4	>9%	106	178	91
B	26	Propylidene phthalide-3 (E)	56014-72-3	95%	159	174	104
B	27	Propylidene phthalide-3 (Z) [e]	94704-89-9	4%	159	174	104
B	28	Acetylcedrene (Vertofix®)	32388-55-9	73%	161	147	119
B	29	Isoeugenyl acetate	93-29-8	>99%	164	149	206
B	30	Amylcinnamaldehyde alpha (Flosal®) (E)	122-40-7	93%	202	129	115
B	31	Amyl salicylate	2050-08-0	100%	120	138	208
B	32	Benzaldehyde	100-52-7	100%	105	106	77
B	33	Benzyl benzoate	120-51-4	>99%	105	212	91
B	34	Benzyl cinnamate	103-41-3	99% [c]	131	192	91
B	35	Benzyl salicylate	118-58-1	100%	91	228	65
B	36	Butylphenyl methylproprional (Lilial)	80-54-6	98%	189	204	147
B	37	Camphor	76-22-2 / 464-49-3	99% [c]	95	152	108
B	38	Carvone	99-49-0 / 6485-40-1 / 2244-16-8	>99%	82	150	93
B	39	Cinnamaldehyde	122-40-7	96%	131	132	103
B	40	Neral = Citral (Z)	106-26-3	49%	69	41	134
B	41	Geranial = Citral E	5392-40-5	50%	69	152	84
B	42	Coumarin	91-64-5	100%	146	118	89
B	43	Damascenone beta (rose ketone-4)	23696-85-7	>99%	177	192	107
B	44	Damascone alpha	024720-09-0	97%	192	123	69
B	45	Damascone beta E	23726-91-2	96%	69	121	190
B	46	Damascone delta (rose-ketone-3)	57378-68-4	94%	192	123	69
B	47	Dimethylbenzylcarbinyl acetate (DMBCA acetate)	151-05-3	>99%	132	117	91
B	48	Eugenyl acetate	93-28-7	98%	164	206	149
B	49	Hexamethylindanopyran (Galaxolide® 1) [d]	1222-05-5	44%	213	228	128
B	50	Hexamethylindanopyran (Galaxolide® 2) [d]	1222-05-6	44%	213	228	128
B	51	Geranyl acetate	105-87-3	>99%	69	136	121
B	52	Hexadecanolactone / Dihydroambrettolide	109-29-5	99%	55	236	41
B	53	Hexylcinnamaldehyde alpha (Jasmonal®)	101-86-0	>99%	216	129	117

B	54	Hydroxycitronellal	107-75-5	96%	59	71	95
B	55	Lyral (minor) [e]	51414-25-6	26%	136	93	59
B	56	Lyral (major)	31906-04-4	73%	136	93	59
B	57	Isomethylionone alpha	127-51-5	88%	135	107	150
B	58	Linalyl acetate	115-95-7	98%	93	136	121
B	59	Methyl salicylate	119-36-8	100%	120	92	152
B	60	Folione	000111-12-6	>99%	95	123	79
B	61	Salicylaldehyde	90-02-8	100%	122	121	65
B	62	Terpinolene	586-62-9	95%	93	121	136
B	63	ISO E® alpha	68155-66-8	63%	191	119	43
B	64	ISO E® beta [e]	54464-57-2	31%	191	119	43
B	65	ISO E® gamma [e]	68155-67-9	5%	191	119	43
B	66	Vanillin	121-33-5	100%	151	81	152
	67	1,4-dibromobenzene	106-37-6	97%	236	238	234
	68	4,4'-dibromobiphenyl	92-86-4	97%	310	152	76

Consecutive gray lines indicate compounds that were used as a mixture of isomers.

[a] Compounds in stock solutions A or B.

[b] Purity determined from the GC-FID area, except if noted.

[c] Purity determined by ^{1}H-NMR.

[d] Isomers not resolved at the baseline: quantified as a whole.

[e] Isomer concentrations that are too discrepant to allow single-run calibrations ~~that are too small to be calibrated in a single run~~ (see Discussion, section 4.3.2).

3.2 Calibration solutions and test samples

For the QMS calibration, two stock solutions, A and B (Table 1, first column), were purchased from Sigma-Aldrich. They contained the allergens without the ISTDs, at a certified concentration of 1,000 mg/kg in methyl pivalate. The set of calibration solutions was prepared by diluting A and B in MTBE, together with 1,4-dibromobenzene and 4,4'-dibromobiphenyl, down to a final analyte concentration of 1, 2, 4, 6, 8 and 10 mg/kg for single-^{2}D, and 2, 5, 10, 25, 50 and 100 mg/kg for dual-^{2}D, with a constant amount of ISTD (1,4-dibromobenzene and 4,4'-dibromodiphenyl at 50 mg/kg for dual-^{2}D and 10 mg/kg for single-^{2}D).

For FID calibration, several stock solutions containing a maximum of seven analytes were prepared at 100 g/kg each. They were further diluted in MTBE to obtain final calibration solutions at 100, 1,000, 2,500, 5,000, 7,500 and 10,000 mg/kg with a constant amount of ISTD (50 mg/kg).

A fragrance model called Lili was formulated by perfumers with 39 ingredients, similar to a real perfume, but it was free of the analytes investigated in this work. Some of these ingredients were themselves mixtures of several compounds. To test the GCxGC method, the fragrance model was accurately spiked with all analytes at three levels, low, mid and high, corresponding to about 2, 50 and 100 mg/kg.

3.3 GC×GC-QMS Instrument

GCxGC analyses were carried out on an Agilent 6890 GC unit coupled with an Agilent 5975 MS detector operating in EI mode at 70 eV (Agilent, Little Falls, DE). The transfer line was set at 280°C. The system was equipped with a two-stage KT 2004 loop-type thermal modulator (Zoex Corporation, Houston, TX) cooled with liquid nitrogen and controlled by Optimode™ V.2 (SRA Instruments, Cernusco sul Naviglio, Italy). The hot-jet pulse time was set at 350 ms and the modulation period (P_M) at 5 s, and the cold-jet total flow was progressively reduced with a linear function from 35% of the mass flow controller at initial conditions to 5% at the end of the run. The hot-jet temperature was programmed from 220°C to 290°C at 3°C/min. The column set consisted of an DB5-MS (Agilent J&W, USA, 30-m long, 0.25-mm I.D., 0.25-μm film thickness) for the first dimension (^{1}D) and an DB1701 (Agilent J&W, USA, 1.4-m long, 0.1-mm I.D., 0.10-μm film thickness) for the second dimension (^{2}D) for GC×GC-MS configuration. The GCx2GC-QMS/FID configuration was built with the same ^{1}D column and an inert three-way "T-Inert" splitter (Agilent G3184-60065) to split the effluent of the first column into the two parallel ^{2}D-columns (two identical DB1701 columns, Agilent J&W, 1.4-m long, 0.1-mm I.D., 0.10-μm film thickness).The first 0.6 m of the two columns were wrapped together in the loop-type thermal modulator slit. One of them was directly connected to the FID, and the outlet of the second one was connected to a deactivated capillary (0.20 m x 0.1 mm ID) with a SilTite® μ-union (SGE, Ringwood, Australia), and then to the MS source (Chromatographic set up in Figure SM-1). The spectrometer was operated under electron impact (70 eV) in scan or SIM mode (see text). The data were acquired by an Agilent MSD ChemStation G1701EA E.02.02.1431 (Agilent Technologies).

Two microliters of each sample solution was injected by using an autosampler ALS 7683B (Agilent), in split mode, with a split ratio of 1:10, at 280°C. The glass liner was filled with deactivated and properly conditioned glass wool. The carrier gas was helium at a constant flow (initial head pressure: 302 kPa). The temperature was programmed from 60°C (1 min) to 240°C (0 min) at 3°C/min, and then to 280°C (5 min) at 25°C/min. After every 40 injections (about 1 week of analytical work), the injector septum and the liner were changed.

The analytes were identified using the NIST MS-Sarch program version 2.2 (Gaithersburg, MD), and a home-made library built with the spectra of authentic compounds listed in Table 1 and acquired using the same instrument. In the case of full scan spectra, the match factor (MF) was calculated. For the spectra made of only three selected 3 ions, the difference between the MF and the reverse-MF (RMF) was determined.

3.4 Signal acquisition method

The full-scan acquisition was made at the fastest scanning rate of 12,500 amu/s, with a mass range of 40-240 uma (5 to 40 min, threshold at 10) and 40-330 uma (40 min till the end of the analysis, threshold at 10) (Figure 1). The solvent delay was set at 5.00 min and the electron multiplier voltage was set in relative mode with the latest Atune value. When the GCxGC-QMS was operated in SIM, no overvoltage of the electron multiplier was applied, the dwell time was 5 ms and the SIM windows had 3 to 15 ions. The FID was heated at 280°C and its sampling frequency was set to 100 Hz.

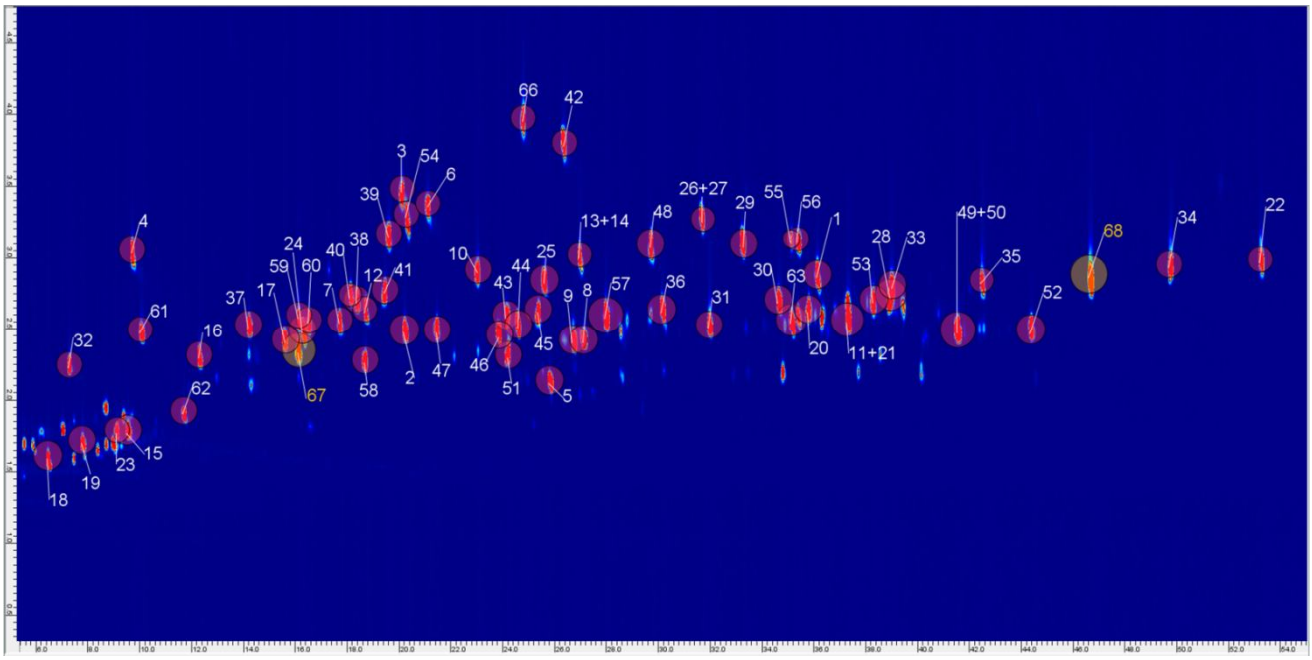

Figure 1. 2D plot of the calibration solution at 25 mg/L with the GCx2GC-QMS/FID system; the plot shows the the QMS signal obtained in full scan acquisition (numbers refer to the analytes listed in Table 1).

4 Results and discussion

In the following discussion, because of the great number of analytes, the results are presented globally as statistical graphs for the sake of clarity.

4.1 Purity and stability of allergen standards

To reduce the time consumption as a result of preparing the calibration solution, we investigated its stability when stored at -80°C in comparison with its initial composition. (Figure 2). Among the 43 analytes free of any overlap, 90% of them deviated by less than 5% over 20 days and by less than 11.5% over 108 days. This includes the variability of the FID (SD of about 5.5% over 150 days in a previous work[40]). Therefore, the storage of calibration solutions under these conditions was considered to be optimal for 3 weeks (median <1%, deviation range within ±5% for 90% of analytes) (Figure 2) and still acceptable for 108 days (range within ±12%).

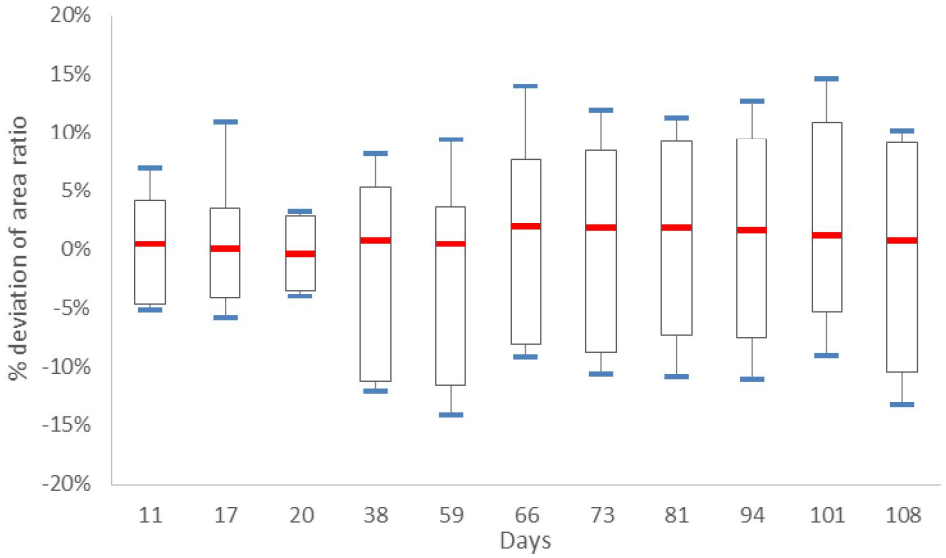

Figure 2. Stability of a calibration solution at 50 mg/kg with the 43 analytes in MTBE stored at -80°C over 108 days. Box plots and blue and red bars represent the 90% deviation range compared to the initial concentration, the extrema and the median, respectively.

4.2 System optimization

In this section, the GCxGC configuration involving parallel dual secondary columns (dual-^{2}D) is compared to the classic one using a single secondary column (single-^{2}D). The MS was operated in scan mode for the former and in SIM mode for the latter.

4.2.1 Peak widths

To quantify so many analytes in such complex media, the system separation power is crucial. This parameter is driven by the peak-widths obtained in both chromatographic dimensions. In this study, half-height peak widths were measured on the 1D raw chromatographic signal. The comparison between the two configurations, i.e., the cryo-modulator in combination with either a single or a parallel dual secondary column(s) (single-^{2}D or dual-^{2}D, respectively), provides prompt information on system separation power. These results were also compared to recently published results of the four-stage flow modulator[21] (Table 2). The latter exhibited larger peaks, presumably due to the absence of cryo-focusing. As expected, the dual-^{2}D produced narrower peaks than did the single-^{2}D because of a flow that was closer to the van Deemter optimum.

Table 2. Peak widths, calibration ranges and corresponding determination coefficients of calibration curves

	Flow modulator[21]	Cryo-modulator-single-^{2}D, SIM	Cryo-modulator-dual-^{2}D		
Detector	MS	MS-SIM	MS-Scan	MS-SIM	FID
Peak width[a] (ms)	600	552	204	312	168
Calib. range (mg/kg)	1-100[b]	1-10	2-100	2-100	100-10,000
Response function	Linear	Quadratic	Quadratic	Quadratic	Linear[c]
Median r^2	09982	0.9978	0.9990	0.9983	0.9998[c]
Min r^2	09910	0.6699	0.9939	0.8384	0.9995[c]
Max r^2	09995	0.9998	1.0000	0.9998	0.9999[c]

[a] Mean peak width values measured on pinene, coumarin and hexadecanolactone.
[b] Using ions different from those of the present work.
[c] Measured on 33 allergens occurring at high concentrations and using a weighing factor of 1/x (see Section 4.3.3 and Table SM-1).

4.2.2 Acquisition mode and repeatability

Because of the better performances of SIM over scan acquisitions that were obtained when developing the method for the 24 allergens using GC-MS, the GCxGC-QMS method was initially developed in SIM mode. However, the recent instruments exhibit improved performances, so that scan and SIM modes were compared again using the dual-^{2}D configuration

and 10 replicates of the calibration solution containing all analytes at 10 mg/kg. The resulting relative standard deviations (RSD) of normalized peak areas were calculated for both modes: in scan mode, 95% of analytes had an RSD below 10%, whereas in SIM mode only 61% of analytes remained below an RSD of 10%. The poor performance of the latter was due to the insufficient rapidity of the detector (minimal dwell time per ion recommended by the manufacturer: 5 msec), whereas many ions (up to 15) had to be monitored in a single SIM window in the case of closely eluting peaks. This led to an insufficient number of data points per ^{1}D and ^{2}D peak. As a consequence, the scan mode was chosen for the rest of this study, thus affording the use of the existing NIST identification algorithm and avoiding loss of the end of a delayed peak when integrating it within the SIM window.

4.2.3 MS stability

In a previous study, we showed that the lack of stability of the GC-QMS response required a recalibration at the beginning of each week.[(3)] This test was repeated here using the GCx2GC-QMS/FID signal (dual-^{2}D configuration) and confirmed the need for a weekly calibration using a recent instrument, as shown by the increase in the median normalized area (≥10%) after 7 days (Figure 3).

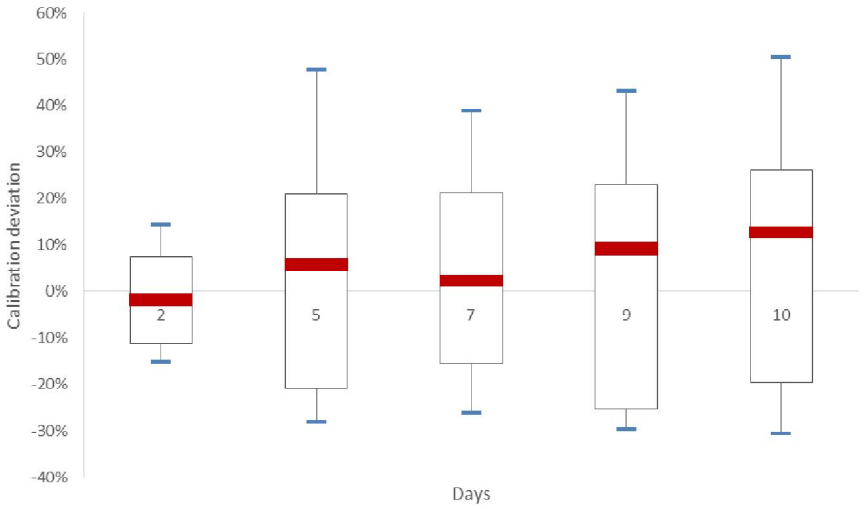

Figure 3. Stability of normalized areas for all analytes over 10 days using the QMS signal of the dual-^{2}D configuration (no tune over the tested period). Box plots and blue and red bars represent the 90% deviation range compared to the initial concentration, the extrema and the median, respectively.

4.2.4 QMS calibration curves (single- and dual-^{2}D)

The objective of these tests was to determine the greatest possible calibration range to minimize the number of sample dilutions when the analyte concentrations were felt to be above the upper limit. The quality of calibration curves was compared by using three conditions: GCxGC-QMS (single-^{2}D) in SIM mode, GCx2GC-QMS/FID (-dual-^{2}D) in SIM mode, and scan mode. The following constraints were imposed on at least 90% of the calibration points: (1) a determination coefficient (r^2) higher than 0.995, (2) biases between the calibration points and the regression line below 20%, and (3) more than 20 significant data points per blob (e.g. abundance >10 S/N). The single-^{2}D configuration gave a range of 1-10 mg/kg for a quadratic calibration that only slightly improved the correlation compared to a linear function. The dual-^{2}D configuration allowed us to extend this range to 2-100 mg/kg by using a quadratic function and the same constraints; however, the calibration biases occurred more frequently at the lowest concentration. This range extension was presumably inherent in the fact that using two ^{2}D-columns in parallel prevented them from overloading and allowed the flow to be closer to optimal conditions. As a consequence, the single-^{2}D configuration was abandoned, and only the dual-^{2}D configuration, associated with MS detection in scan mode, was used to pursue this work.

4.2.5 FID calibration curves

As recalled in the previous paragraph, an efficient application of the present method in quality control requires minimization of the number of sample dilutions. In the case of the dual-^{2}D, the parallel FID detection gave an extended quantification range above that of QMS. For low concentrations, the use of a selective detector such as an MS is compulsory to overcome the quantification of coeluted compounds, and thus the GCxGC-MS system is tridimensional. In contrast, GCxGC-FID consists of only two dimensions and it is insufficient to overcome the coelutions observed at a level of 50 mg/L in a model fragrance.[(15)] From the SCCS list of allergens, only 33 of them sometimes occur above 10% in fragrances. Most of them (29) are natural constituents of essential oils (see the list in Table SM-1). The number of abundant peaks in a given formulation is limited, therefore, the coelutions between them are not frequent; their coelution with minor peaks are not significant and can be detected using the MS signal prior to FID quantification. For these 33 analytes, linear curves were drawn with six calibration points from 100 to 10,000 mg/kg, but many residuals at 100 mg/kg were in excess of 50% because of the leverage effect of high concentration points. Therefore, a weighing factor of 1/x was applied to the calibrations, which gave r^2 in excess of 0.9996 and all residuals below or equal to 10% (Figure 4). This potentially extends the quantification range to 2-10,000 mg/kg in a single run by combining the MS and FID detections.

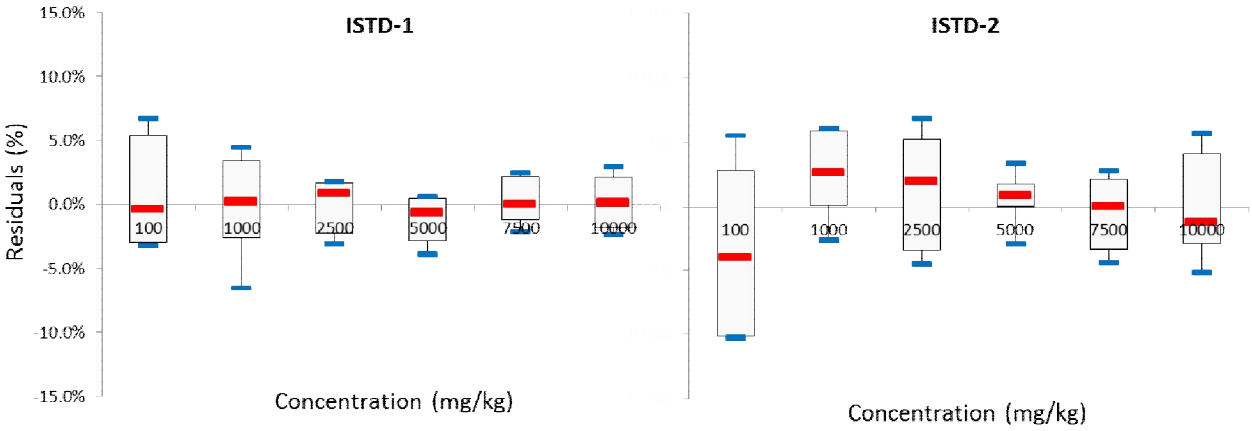

Figure 4. Calibration residuals of analytes (GCx2GC-QMS/FID configuration and based on FID signal) when referring to ISTD-1 (1,4-dibromobenzene) or ISTD-2 (4,4'-dibromodiphenyl). Box plots and blue and red bars represent the 90% range of residuals, the extrema and the median, respectively.

4.2.6 Identification of analytes

When a multianalyte analysis is performed in a complex matrix, the positive identification of target peaks in the quantification run is a compulsory good practice.[41] It avoids the confusion with a neighboring peak, and it alerts the analyst in case of an impure peak due to a coelution. In the present work, the data were processed with GC Image and only the NIST identification algorithm was available. The latter is designed to identify full-scan spectra, and there is no integrated alternative to identify non-full spectra, such as the rules of the European Directive,[42] or the calculation of the Q-value.[3] Therefore, the following strategy was arranged. For the automated processing of GCxGC-QMS files with GC Image, we selected a retention time tolerance of 1%, associated with a low minimum value for the match factor (>700), to avoid analyte missing. Then, the difference between MF and RMF was calculated: the MF determines the identity between unknown and the library spectra, using all ions of the former, whereas the RMF ignores any peaks in the unknown that are not in the library spectrum.[43] The peak was considered to be free of interference below an absolute difference of 50, as experimentally determined by scrutinizing the spectra of a large set of analytes spiked in the matrix at various concentrations.

When it exceeded 50, the three specific ions of the analyte were selected (Table 1), and the MF was recalculated on the basis of these three ions. When the MF was higher than 900, the three

ions were considered to be free of interference and the quantification using Q1 was validated. On the other hand, the final analyte quantity was chosen as the minimal value resulting from the three ions, Q1, Q2 and Q3. The latter conclusion was based on the fact that the most frequent cases of bias were due to coelutions leading to overestimations. When all allergens at 50 mg/kg were spiked in the Lili matrix, 50 of 60 analytes were positively identified, with ΔMF < 50 (ΔMF = | MF-RMF |). This strategy is summarized in the decisional tree (Figure 5) and illustrated in Section 4.3.1.

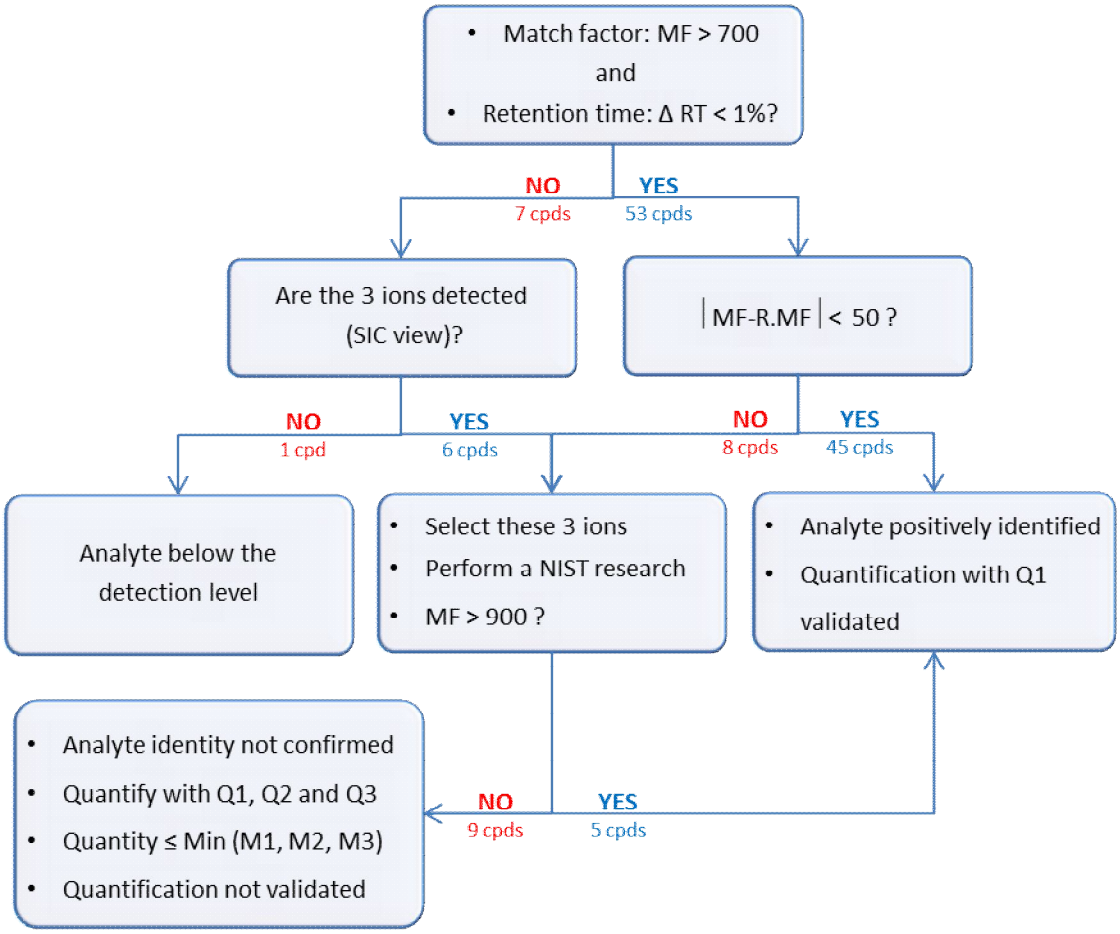

Figure 5. Decisional tree used to validate the quantitative result by confirming the analyte identity and purity. The number of compounds (cpds) indicated on the arrows refers to the quantification of Lili spiked with all allergens at 50 mg/kg (see also text and Table 3). In this experiment the analytes corresponding to footnotes *d* or *e* in Table 1 were quantified as a whole or not quantified, respectively.

To test the decisional tree, we spiked a fragrance model, Lili, at 50 mg/kg with all allergens and quantified them. The composition of Lili was intentionally complex (39 ingredients but *ca*. 150 GC peaks), mainly because one constituent, hedione, occurring at a concentration of 37%, gave rise to major coelutions with some allergens (Figure SM-2). All analytes, except Lyral, eluted within a tolerance of 1%, and so they matched the first identification point. Of the 59 remaining analytes, 53 had an MF in excess of 700 and were considered to be positively identified (two independent criteria). Of these 53 analytes, 45 exhibited a difference $\Delta MF < 50$, indicating that their spectra were free of interference and so the corresponding quantitative result was validated. The three specific ions were selected for each of the 15 compounds with MF <700 or $\Delta MF \geq 50$ (in bold in columns "MF Full spectrum" and "ΔMF," Table 3): five analytes gave an MF >900, indicating that the "reduced" spectra were free of interference (non-bold in column "MF 3 ions," Table 3). This result validated the quantities of these five additional compounds, leading to a total of 50 (83%). The amounts of the remaining analytes were determined from their three specific ions, and the minimum of these three values was selected as a non-validated concentration (underlined values, column "Final bias," Table 3). The major isomer of Lyral showed a tailed peak that was responsible for the bias of its retention time and poor integration. It was strongly underestimated, possibly because of its coelution with hedione, the major Lili constituent, which gave rise to a detector saturation. As a consequence, its minor isomer was presumably below the quantification limit and was not considered in the statistics. Globally, the 59 other analytes were quantified; among them, 90% were within the tolerance limit of 30%, and only one compound (Lyral) exhibited a bias of more than 90%.

Table 3. Application of the decisional tree to the Lili model, spiked at 50 mg/L with all allergens

Compound	ΔRT	MF Full spectrum	ΔMF	MF 3 ions	Final bias
Methyl salicylate	0.5%	**168**	**206**	**857**	**-49%**
Geraniol	0.0%	**664**	**148**	**606**	-16%
Anethol	0.8%	793	**128**	**853**	5%
Damascenone beta	0.0%	738	**110**	**546**	-7%
Isomethyionone alpha	0.3%	853	**53**	**781**	3%
Eugenyl acetate	0.3%	777	**64**	957	3%
Propylidene phthalide	1.3%	**206**	**262**	988	**-61%**
Amyl salicylate	0.8%	817	**97**	966	30%
Isoeugenyl acetate	1.3%	577	**105**	972	-3%
Amylcinnamaldehyde	0.2%	847	**61**	**390**	0%

alpha					
Galaxolide 1	0.4%	**448**	**168**	975	-6%
Benzyl cinnamate	0.2%	830	**91**	**546**	**-35%**
Sclareol	0.2%	871	**54**	**376**	-11%
Lyral (Major)	**4.0%**	n.m.	n.m.	**655**	**-96%**
Lyral (Minor)	-	n.m.	n.m.		n.q.

Only the analytes with ΔMF > 50 are listed. The values that do not pass the decisional tree criteria are in bold. Underlined biases result from the choice of the minimal amount among Q1, Q2 and Q3. n.m.: no match factor; n.q.: not quantified.

4.3 System performances

4.3.1 QMS quantification of a spiked model fragrance

The fragrance model Lili was spiked with all allergens at three levels, 2, 50 and 100 mg/kg, and these samples were used to evaluate the performances of the GCx2GC-QMS/FID (dual-^{2}D) method. The identity of analytes was checked by using the NIST match factor, as described earlier. The performance of the GCx2GC-QMS/FID configuration operating in full scan mode was compared with the usual GC-MS procedure in SIM, formerly published for the analysis of the 24 allergens,[5, 6] and extended to the new list of allergens by IFRA,[44] using two runs of 22 and 36 compounds, corresponding to the stock solutions A and B of Table 1. As a result, the dual-^{2}D configuration greatly increased the proportion of analytes quantified with a bias of less than 30% (Figure 6). In addition, the results of the injection on two different phases, made in two separate runs in GC-MS, were obtained in a single run in GCxGC-MS.

The proportion of analytes quantified with a given bias was similar at mid and high spiking levels for both GC-MS/SIM and GCx2GC-QMS/FID in full scan. At these levels, the latter configuration showed 85% and 90% of analytes quantified with a bias lower than 30%. The greatest biases were due to the coelution with the ingredient hedione, occurring at 37% in the Lili formula. Hedione overloaded the MS detector and led to a distortion of coeluted analyte spectra.

The recoveries of the sample spiked at a low level were frequently overestimated because of the occurrence of isobaric interference with ions from model fragrance components. The effect of these interferences on the low spiked amount significantly impaired allergen quantification below 50 mg/kg.

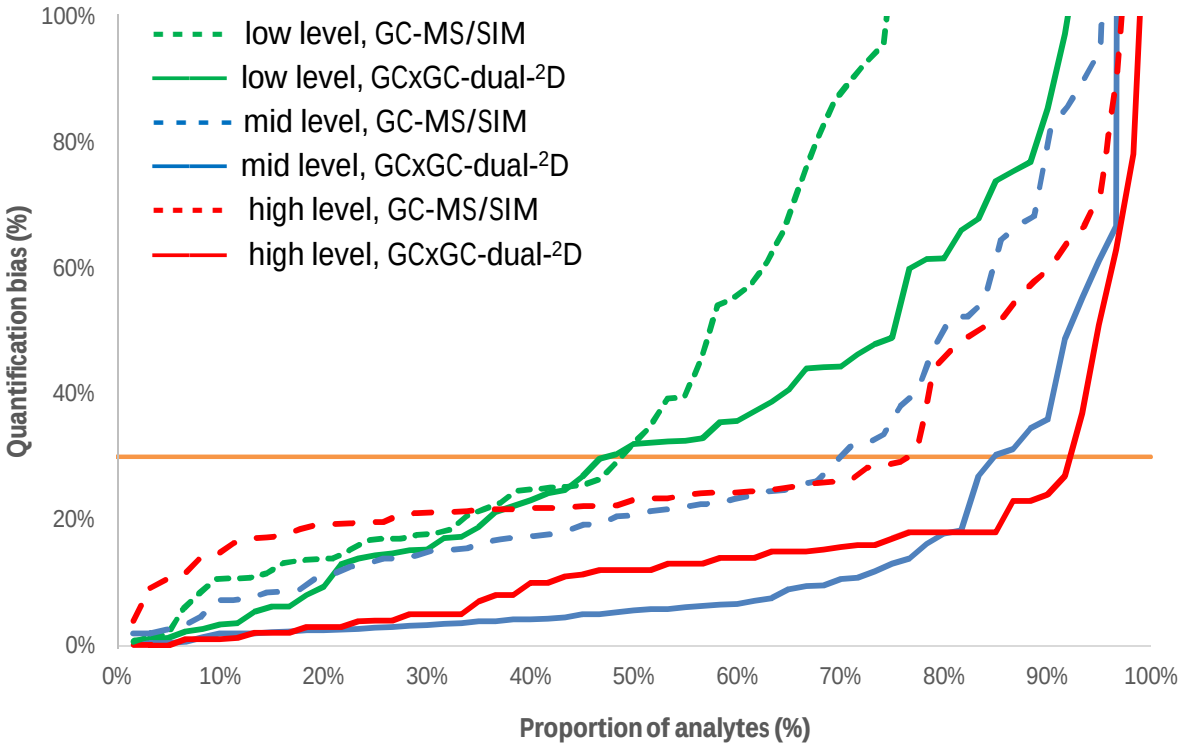

Figure 6. Absolute quantification biases as a function of the proportion of analytes using GC-MS/SIM and GCx2GC-QMS/FID in full scan at three spiking concentrations.

Globally, the proportion of analytes quantified with a bias of less than 30% was increased moderately at low concentration level, and greatly at mid and high levels. All of these results will be further tested under validation conditions by using the accuracy profile approach in a forthcoming paper (E.B., P.M., A.C., unpublished data, 2017).

4.3.2 QMS quantification of minor isomers

The minor isomers of farnesol, isoeugenol, propylidene phthalide-3 (Z), terpineol (Figure SM-3) and iso-E-super are not sufficiently pure for calibration. Calibrating them up to 100 mg/kg using the mixture of the major isomer is unrealistic, because it would lead to a huge peak of the latter that would interfere with the peaks of other analytes. Therefore, only the isomerically pure standards of major isomers were added in the stock solutions A and B, and their calibration curves were used to quantify the minor isomers. The proportion of each isomer was determined

by GC-FID because their response factors were identical.[45] These proportions were then used to calculate the concentration of each isomer when calibrating with the isomeric mixture for the MS detector.

Exact solution: This is illustrated here by using alpha- and gamma-terpineol (αT and γT, respectively) as the major and minor isomers. The GC-FID analysis indicates that αT and γT represent 66% and 22% in the isomeric mixture, respectively. The QMS calibration gave two quadratic curves (Equations 1 and 2):

$$\overline{C}_{\propto T} = 0.0602\overline{A}_{\propto T}^{2} + 1.0381\overline{A}_{\propto T} - 0.0164 \qquad \text{(Equation 1)}$$

$$\overline{C}_{\gamma T} = -0.0146\overline{A}_{\gamma T}^{2} + 0.4873\overline{A}_{\gamma T} - 0.0006 \qquad \text{(Equation 2)}$$

With: $\overline{C}_{\propto T}$, $\overline{C}_{\gamma T}$, $\overline{A}_{\propto T}$, $\overline{A}_{\gamma T}$, the normalized concentrations and areas of αT and γT.

Because the same isomeric mixture was used to build both calibration curves, the isomeric proportions were identical at all concentrations and so: $C_{\gamma T} = \lambda C_{\propto T}$. This factor λ was determined by minimizing the sum *S* of differences between squared calculated and spiked concentrations of γT using the Excel Solver (Equation 3).

$$S = \sum_{i=1}^{i=n}\left[\left(\overline{C}_{\gamma T,i}^{(real)}\right)^{2} - \left(\lambda\langle 0.0602\overline{A}_{\gamma T,i}^{2} + 1.0381\overline{A}_{\gamma T,i} - 0.0164\rangle\right)^{2}\right] \qquad \text{(Equation 3)}$$

With: *n*, the number of calibration points and $\overline{A}_{\gamma T,i}$ and $\overline{C}_{\gamma T,i}^{(real)}$, the normalized areas and real concentrations of γT in calibration solutions, respectively.

The resulting value of $\lambda = 0.4480$ gives the concentration of the minor isomer γT when using the calibration curve of the major one, αT, which allows one to draw a virtual calibration line of γT (Figure 7, dotted line). The residuals of the virtual calibration points versus the real concentrations are reported in Table 4, lines "In MTBE]".

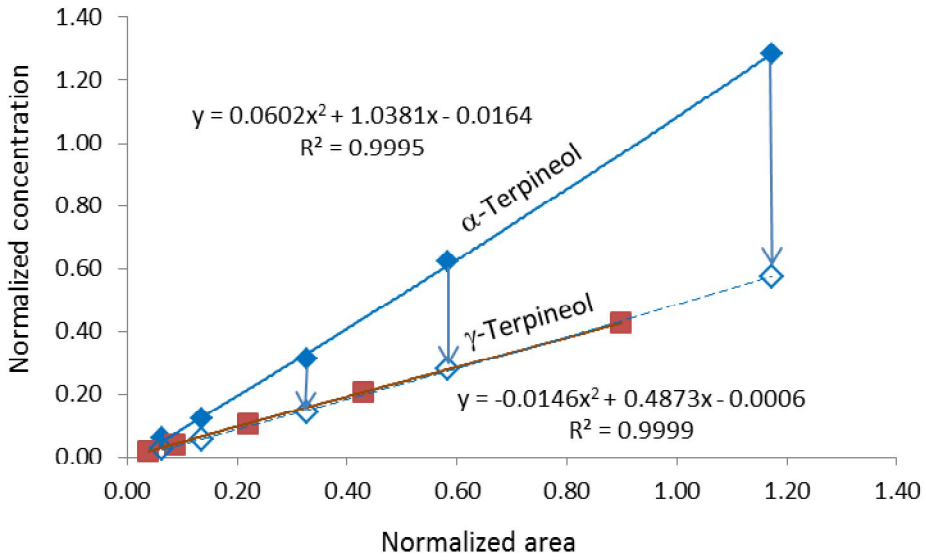

Figure 7. Linear calibration curves resulting from the injection of the terpineol mix (exact solution). The blue dotted line (---) and empty diamonds (◇) represent the virtual calibration curve of γT recalculated from those of αT (blue diamonds◆). Dark-red squares indicate the γT experimental calibration (■).

Simplified solution: As a quicker way to routinely quantify the minor isomers, the following approximation was tested: over the short range in common with that of the major isomer (from 2 ppm up to the highest level corresponding to 100 mg/kg of the major isomer), the calibration curve was considered to be linear and its offset to be negligible. Under such conditions, the slopes of the calibration curves represent the relative response factors (RRF) of both isomers: $\overline{m}_{\gamma T} = RRF_{\gamma T}\overline{A}_{\gamma T}$ and $\overline{m}_{\propto T} = RRF_{\propto T}\overline{A}_{\propto T}$ measured with the MS, and λ is the ratio of slopes $\lambda = \frac{RRF_{\gamma T}}{RRF_{\propto T}} = \frac{\overline{m}_{\gamma T}}{\overline{A}_{\gamma T}}\frac{\overline{A}_{\propto T}}{\overline{m}_{\propto T}}$. The resulting values of λ for the calibration solution C1-C4 of the terpineol mixture are reported in the corresponding line of Table 4.

Table 4. Residuals of the virtual γ-T calibration, using the λ optimization from quadratic curves and individual calibration solutions C1-C4 (lines "in MTBE", exact solution). Quantification biases, using the virtual γ-T calibration, of four Lili samples spiked with the terpineol mixture (Lines "In Lili", simplified solution).

	λ calculated from: [α-T]= [γ-T]=	Quadrat. curves	C1 81 mg/kg 27 mg/kg	C2 41 mg/kg 14 mg/kg	C3 21 mg/kg 7 mg/kg	C4 8 mg/kg 3 mg/kg
	λ →	0.4480	0.4330	0.4522	0.4942	0.5279
In MTBE	[C1]: [γ-T] = 27 mg/kg	2%	-2%	3%	12%	20%
	[C2]: [γ-T] = 14 mg/kg	-5%	-8%	-4%	5%	12%
	[C3]: [γ-T] = 7 mg/kg	-8%	-11%	-7%	2%	9%
	[C4]: [γ-T] = 3 mg/kg	-18%	-21%	-17%	-10%	-4%
[γ-T] in Lili	[C1]: [γ-T] = 27 mg/kg	-3%	-6%	-2%	7%	14%
	[C2]: [γ-T] = 11 mg/kg	3%	-1%	4%	13%	21%
	[C3]: [γ-T] = 7 mg/kg	5%	1%	5%	15%	23%
	[C4]: [γ-T] = 3 mg/kg	-3%	-10%	-6%	3%	10%

Quantification in the Lili matrix: The simplified solution was tested by spiking the Lili matrix with the mixture of terpineol isomers at four different levels in a solvent. It was quantified by using the virtual calibration curve of γ-T, i.e. the calibration curve of α-T associated with the correction factor λ determined from the injection of only one of the calibration solutions C1-C4. All results were satisfactory, but the determinations with a single injection led to more accurate quantifications by using the two most concentrated terpineol solutions (C1-C2) than by using the least concentrated solutions (C3-C4) because of a possible interference with the background (Table 4, lines "In Lili).

4.3.3 FID quantification of spiked vetiver oil

The need of quantifying very abundant analytes by FID mostly occurs in the case of essential oils. The applicability of the dual detection was thus tested by spiking a vetiver essential oil free with some of the 29 naturally occurring analytes from the 33 abundant ones mentioned in section 4.2.5. Simultaneously spiking the 29 constituents in an essential oil at all concentrations would not be feasible, because the highest level would correspond to a total of 29% in the tested oil. Therefore, seven constituents were randomly selected and spiked in vetiver oil (Figure SM-4) at four different levels and quantified by using 1,4-dibromobenzene as an ISTD. The weighed

calibration curves are described in the section “FID calibration curves.” All biases were less than 10%, except for sclareol at 100 and 500 mg/kg (Table 5).

Table 5. Biases resulting from the FID quantification of a spiked vetiver oil using the GCx2GC-QMS/FID system using 1,4’-dibromobenzene as an ISTD

Compound	9000 mg/kg	5000 mg/kg	500 mg/kg	100 mg/kg
Pinene alpha	-3%	-2%	-5%	-4%
Linalool	-3%	-2%	-5%	-5%
Anethole	-1%	0%	-3%	-2%
Eugenol	0%	0%	-3%	-1%
Vanillin	1%	2%	-3%	2%
Eugenyl acetate	1%	2%	1%	10%
Sclareol	3%	5%	11%	51%

Using the second ISTD (4,4’-dibromodiphenyl), the biases were higher (6-21%) and up to 39% at 100 mg/kg. Such results suggest the applicability of the FID branch of the dual parallel configuration to the quantification of major constituents in natural raw material, thereby extending the quantification range of the system.

5 Conclusions

The present results clearly demonstrate the feasibility of single-run quantification of the 54 chemically defined allergens and their isomers by comprehensive 2D-GC coupled with both QMS and FID in parallel, compared with the previous GC-MS approach, which required an injection on two different GC phase polarities and sample dilutions to fit the MS calibration range. This work is the first fully developed quantitative method based on dual-^{2}D configuration, which provides better system loadability and optimization of ^{2}D flows. The combination of the calibration range of both detectors affords quantification of investigated analytes from 2 to 10,000 mg/kg while maintaining satisfactory calibration residues. A new strategy, summarized in a decisional tree, enables the confirmation of peak identification and purity to validate quantifications in complex matrices. A rapid and simple quantification procedure of minor isomers is also proposed for routine analyses. The validation of the current method will be the purpose of a forthcoming paper.

5.1 Acknowledgements

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