Hyphenation of Quadrupole MS to GC and Comprehensive Two-Dimensional GC for the Analysis of Suspected Allergens: Review and Improvement

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Abstract

This survey reviews papers that have been previously published on the quantitative analysis of suspected allergens. The routine gas chromatography (GC)–mass spectrometry (MS) method allows their evaluation in most fragrances, but the application of an automated data treatment sometimes leads to over- or underestimations when target compounds are coeluted or shifted because of the presence of other fragrance ingredients. In such cases, an appropriate retreatment of data generated by the routine analysis is proposed to better estimate these shifted or coeluted peaks. A second and more sophisticated approach, based on comprehensive bidimensional GC hyphenated to quadrupole MS, overcomes coelution problems. However, its use is still time consuming because of the lack of a commercial program. In this work, a software prototype is tested to reprocess the data. It dramatically shortens the data treatment and offers good quantitative results.

Introduction

For several years, allergens in consumer products have become an important concern for the European community. Various compounds are involved: metals, plastics, dyes, colophony, etc. Among them, some fragrance compounds are suspected to elicit skin reactions using patch tests. According to the 7th Amendment of the European Cosmetics Directive (1), 26 substances will have to be indicated on the labelling if they are present at concentrations exceeding 0.001% in cosmetics that are intended to remain on the skin or 0.01% in those that are rinsed off the skin. Of these 26 substances, 24 are chemically defined volatile compounds (Table I).

These new rules can only be applied and policed if a suitable analytical method allows their quantitation. This is not only valid for consumer products but also for intermediate products such as fragrance concentrates or essential oils, as monitoring the amounts of these 24 compounds now becomes part of the quality control of the perfume industry. It must be emphasized that analyzing allergen traces in the presence of hundreds of fragrance ingredients is often a tricky task. Therefore, the Analytical Working Group of the International Fragrance Association (IFRA) has developed a routine gas chromatography (GC)–mass spectrometry (MS) method that is described below (2). Another approach will also be reported in this paper. It is based on the dramatic peak capacity improvement caused by comprehensive-bidimensional GC (GC×GC), a very recent analytical technique. For both approaches, some improvements of the data treatment will be discussed.

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Discussion

Routine GC–MS analyses

Initial literature

For the routine analysis of fragrance extracts, a single method should be used to evaluate all possible allergens. The method should also be based on instruments that are commonly found in

Table I. List of the 24 Suspected Allergens

Name	CAS reg. no.	Name	CAS reg. no.
Amylcinnamic alcohol	[101-85-9]	Farnesol	[106-28-5]
Amylcinnamic aldehyde	[122-40-7]	Geraniol	[106-24-1]
Anisyl alcohol	[105-13-5]	Hexylcinnamic aldehyde	[101-86-0]
Benzyl alcohol	[100-51-6]	Hydroxycitronellal	[107-75-5]
Benzyl benzoate	[120-51-4]	Isoeugenol	[97-54-1]
Benzyl cinnamate	[103-41-3]	Butylphenyl methylpropional	[80-54-6]
Benzyl salicylate	[118-58-1]	Limonene	[5989-27-5]
Cinnamic alcohol	[104-54-1]	Linalool	[78-70-6]
Cinnamic aldehyde	[104-55-2]	Hydroxyisohexyl-3-cyclohexene carboxaldehyde	[31906-04-4]
Citral	[5392-40-5]		
Citronellol	[106-22-9]	Methyl 2-octynoate	[111-12-6]
Coumarine	[91-64-5]	α-Isomethylionone	[127-51-5]
Eugenol	[97-53-0]		

the fragrance industry. Only two papers have reported such a possibility prior to the IFRA work. The first one involves the identification of target compounds using a GC–MS, prior to their quantitation, by a GC equipped with a flame-ionization detector (FID) (4). This latter step does not allow the evaluation of allergens coeluting with other perfume constituents. To overcome this difficulty, in a later report, the same authors directly made this evaluation from specific ions extracted from the full-scan data file (5). This is not consistent with recommended procedures for GC–MS quantitation that should be achieved in simultaneous single ion monitoring (SIM) mode (6). A second publication follows these guidelines and claims recoveries in the 98–106% range with a detection limit of 2 mg/L (7). Although two ions were used for the quantitation, it is unclear whether their abundance ratios were used to check the peak identities as recommended in SIM experiments (8). The method was tested using a fragrance spiked with five allergens at a level of 20 mg/L. Quantitation showed that the method required significant improvements as none of the added compounds was satisfactorily evaluated (Table II).

IFRA method (2,3)

A routine method has been developed for use by all partners of the perfume industry: suppliers of raw materials, fragrance companies, manufacturers of perfumed end products, and public laboratories. As GC–quadrupole-MS instruments are commonly used by many of these partners, this technique was chosen because its high selectivity and quantitative performances are known. The chromatogram is divided into retention time windows, each of them successively monitors one or two target compound(s). Each target compound is characterized by one quantitation ion and two "qualifier" ions. The abundance ratios between qualifiers and the quantitation ions allow a check of the identity of the suspected allergen, according to the following formula:

Table II. Quantitation of Five Spiked Compounds at a Level of 20 mg/L*

Compound	Spiking (mg/L)	Found (mg/L)	Q	Comment	
Limonene	0	9.3	89	Not added	
Linalool	20	49.7	1	Not recognized	Wrong amount
Anisic alcohol	20	123.5	95	Recognized	Wrong amount
Cinnamic alcohol	0	74.8	96	Not added	
Eugenol	20	90.5	39	Not recognized	Wrong amount
α-Isomethylionone	20	9.2	18	Not recognized	Wrong amount
Benzyl benzoate	20	7.9	56	Not recognized	Wrong amount

* In the fragrance sample SVB, using 2 ions/compound. Target peaks are recognized when Q is greater than 89.

$$Q = 100 - \frac{\sum_{i=1}^{i=n} \left(100 * |r_i - r_i'|\right) (\mathrm{In}[100 r_i + 1])^2}{21.3 * \sum_{i=1}^{i=n} r_i} \qquad \text{Eq. 1}$$

where n is the number of ions per compound, r_i is the reference peak area ratio, and r_i' is the observed peak area ratio

A Q-value between 90 and 100 indicates a positive recognition of the target peak. A lower value indicates that either the quantitation ion belongs to another compound or coelutes with another analyte.

To overcome difficulties that are exemplified in Table II, all analytical conditions were optimized. The best chromatographic separations were observed with a (50%-phenyl)-methylpolysiloxane column. However 100%-methylpolysiloxane phases under high flow and temperature rate conditions also gave satisfactory resolutions. In addition, this latter type of column is widely available in quality control (QC) laboratories. The injector cleanness was identified as a critical issue, because fragrance concentrates often contain low-boiling ingredients; calibrating the instrument without cleaning the injector led to the wrong quantitations (Figure 1, dirty injector).

Citronellal was previously proposed as an internal standard (7), but it is known to be unstable. Brominated internal standards were used because they are stable, normally absent from fragrance compositions, and exhibit characteristic ions. Isooctane and *o*-fluorotoluene are suitable solvents to dilute the sample and calibration solutions, depending on the polarity of the sample.

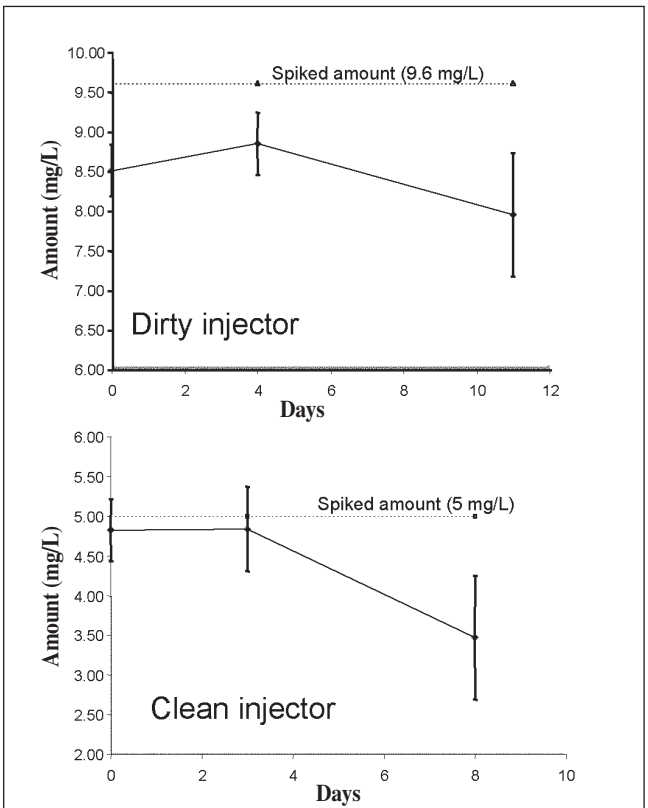

Figure 1. Calibration alterations caused by injector pollution (top) and to the drift of the MS detector (bottom) [adapted from Chaintreau et al. (2)].

Conversely, low-boiling and protic solvents were rejected as the former readily evaporate and protic solvents (e.g., ethanol) promote reactions of allergens (e.g., acetal formation). Both phenomena alter the concentration of standard solutions. The stock solution containing the allergens was stable for 1 month in the freezer or 2 months in the refrigerator if carbonyl and noncarbonyl compounds are stored separately.

The calibration validity was observed to be potentially altered by two causes. First, perfumes may contain low-volatile ingredients that remain in the injector. As a consequence, the dirty injector gives rise to evaluations of allergens that deviate from the truth even on the same day of the calibration (Figure 1, dirty injector). Second, the MS calibration validity is limited in time. Under the present conditions, it was observed to be only valid for 1 week (if calibrated on Monday), then a drift occurred (Figure 1, dirty and clean injector). This phenomenon is documented little in the literature (9).

The method was tested by spiking three fragrances with 50 or 100 mg/L of various target compounds. The mean recovery was 100.5% with a coefficient of variation of 16%. A second injection in scan mode may be required to check the presence or absence of a peak exhibiting a Q-value below 90 (e.g., benzyl benzoate in Table II). In spite of the GC–MS selectivity, coelutions of target compounds with fragrance ingredients containing isobaric ions sometimes occur. In such a case, a third injection of the same sample using a different GC phase must be performed, which necessitates a recalibration of the instrument. This gives rise to time-consuming analyses for a single sample. To shorten this time, either an improved data treatment of quantitative GC–MS results may be used (see the following section), or a more selective technique must be chosen to obtain pure GC peaks (see the GC×GC–MS section).

Solving coelutions and peak shifts in GC–MS

Up to now, the previously mentioned routine GC–MS procedure is presumably the most suitable method for QC laboratories. With most instruments, the data treatment can be automatically achieved by the GC–MS workstation. However an "automated interpretation" may have some limitations, especially when peaks exhibit Q-values below 90. Then a "human interpretation" is required. The IFRA procedure thus recommends an injection in scan mode in addition to the SIM analysis. Almost all cases behind the software capabilities are attributable to either coelutions or a shift of the retention time of target compounds.

Table III. Examples of Target Compound Quantitations in Real-Life Samples Using the Lowest Determination from Three Ions*

Sample & target compound	Spiking amount	Ion 1	Ion 2	Ion 3
TPNL	–	*m/z* = 93	*m/z* = 71	*m/z* = 121
Linalool	0	1441 (10)	9 (1)	35 (1)
	55	1498 (13)	60 (1)	91 (1)
BDN	–	*m/z* = 108	*m/z* = 107	*m/z* = 79
Benzyl alcohol	0	32 (95)	3101 (90)	275 (89)
	27	60 (98)	1591 (96)	515 (97)
PPEE	–	*m/z* = 206	*m/z* = 150	*m/z* = 135
α-Isomethylionone	0, no t_R† adjustment	3.6 (1)	12 (1)	0.6 (1)
	100 + t_R adjustment	96 (96)	111 (99)	97 (97)

* Amount in mg/L, Q-values in brackets.
† t_R = retention time.

Coelution

Coelution is the most frequent case. Because of the variability of fragrance compositions, a target compound can elute with another analyte exhibiting an ion identical to the one used for the quantitation. Therefore, it gives rise to an overestimation of the target compound. From such an observation, a more elaborate interpretation of existing acquisition files may often overcome the previously mentioned problem, without requiring a recalibration and reinjection of the fragrance using a different column.

As, starting from a single injection, three ions per compound are monitored at the same time, three calibration curves may be drawn for each compound. Assuming that only overestimations attributable to coelutions alter the peak areas, the lowest amount that is deduced from the three calibration curves should be the closest to the real quantity.

Two cases exemplify how this approach may help interpretations of the result (Table III). In the "TPNL" sample, the evaluation of its linalool content was suspected to be erroneous according to its first ion because of a low Q-value (Q = 10). The minimum evaluation from these three ions was 9 mg/L. After having spiked TPNL with 55 mg/L of linalool, it was requantitated. The linalool amount increased to 57, 51, and 56 mg/L according to ions 93, 71, and 121, respectively. This confirmed the validity of the minimum value found with *m/z* of 71.

The second case exemplifies a different situation. For "BDN", the Q-value of the first ion was satisfactory. However, Q-values of ion 2 and 3 were at the positive identification limit (89–90), but the quantities strongly differed. Here again, spiking the sample with the lowest amount found from the three ions confirmed the validity of the evaluation based on the first ion.

Shifted peaks

This phenomenon is observed when a target peak is eluted just after a much more abundant constituent of the fragrance. In the third example ("PPEE"), α-isomethylionone is apparently absent from this fragrance. However, the full-scan analysis of the same product shows the elution of a major constituent just before the α-isomethylionone window. This fact generally delays the elution of following peaks. After having spiked the sample with this allergen, the peak apex was shifted out of the defined retention time window and was not quantitated. The retention time window was readjusted to the correct retention time, and the quantity was recalculated from the same data (Table III). The final result gave a quantity of approximately 100 mg/L, which was in agreement with the spiked amount. The real α-isomethylionone quantity in PPEE was then considered to be negligible.

These three real-life examples, selected from the day-to-day practice of QC laboratories, shows that limitations of automated

reporting procedures may often be overcome by a "manual" interpretation of existing results by a trained analyst.

Comprehensive GC

The occurrence of the previously mentioned coelutions in GC–MS is inherent in the restricted peak capacity of chromatographic columns compared with the great number of possible fragrance constituents. The use of GC–MS under chemical ionisation conditions represents a valuable tool to overcome this difficulty as it significantly increases the detection selectivity. This alternative has been reviewed elsewhere (10). Multidimensional GC may solve a few coelutions in a given analysis (11), but the number of achievable heart-cuts is still too limited for a general application when the sample composition is highly variable. A decade ago, Phillips introduced a new technique called comprehensive bidimensional chromatography (also referred as GC×GC) (12). The usual configuration consists of a first normal capillary column coupled to a short and small-diameter column with a different phase. Using a modulator based, for instance, on a moving cryo-trap, peaks eluting from the first column are successively trapped and pulsed in the second one every 3–5 s and subsequently separated. The scheme of the instrument used to analyze allergens is shown in Figure 2, and more details about GC×GC may be found in various reviews (13,14).

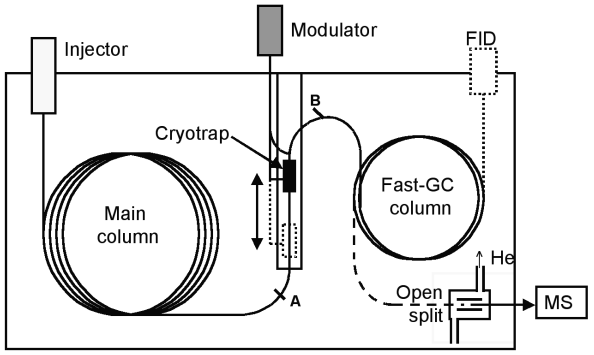

Figure 2. Comprehensive GC with either MS (long dotted line) or FID (short dotted line) detection and using a longitudinally modulated cryogenic system (15).

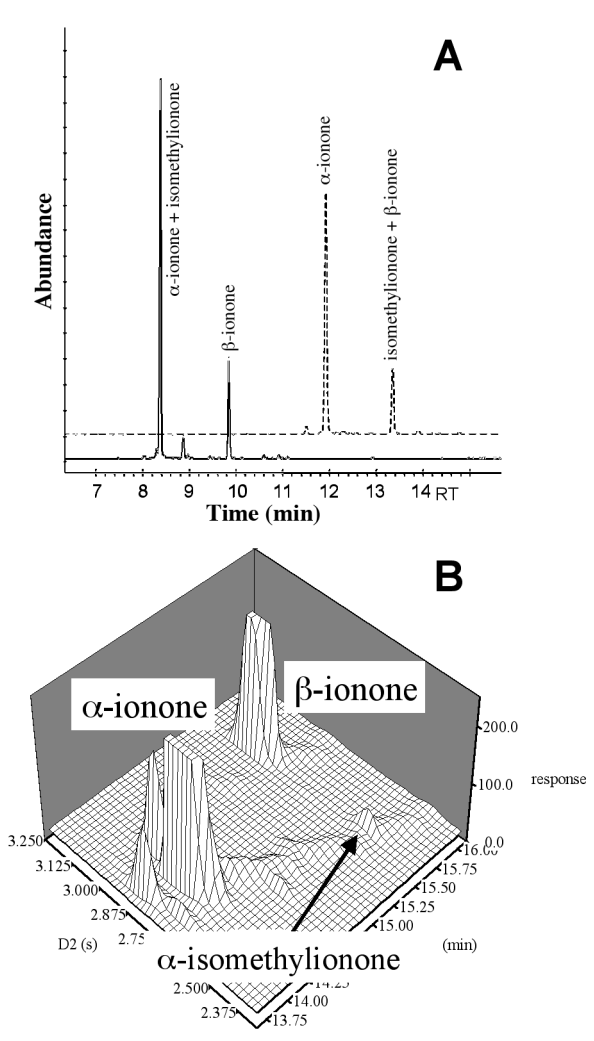

Figure 3. Analysis of a violet-like fragrance using: (A) a single PDMS column (dotted line) or PEG column (continuous line) or (B) a PDMS×PEG configuration [adapted from Shellie et al. (16)].

GC×GC–FID (16)

In contrast to the separation optimization of all allergens from each other by GC–MS, full separation was achieved as soon as the first GC×GC injection using a combination of polydimethylsiloxane (PDMS)- and polyethylene glycol (PEG)-type columns for the first and second dimension, respectively. To exemplify the separation of the GC×GC system, a violet-like sample was used. Its analysis using a single column system led to perfect coelution either with a PDMS- or a PEG-type column (Figure 3A). Reanalyzing the same sample with a combination of the same columns gave a clear resolution of the α-isomethylionone peak (Figure 3B).

The calibration curves were linear in the range of 20–1000 mg/L (r^2 > 0.995) for all compounds of interest. However, coelutions still occurred when analyzing allergens in complex fragrance concentrates. This limitation is clearly illustrated in the analysis of the "SVB" sample (168 ingredients) spiked with 5 target compounds. Linalool and anisyl alcohol could not be quantitated by comprehensive GC×GC–FID, as their peaks were not clearly discernable within their 2D retention area (Figure 4). The same two compounds could also not be evaluated by GC–MS using the same column as the first GC×GC dimension (PDMS) (Table IV).

GC×GC–MS

The occurrence of coelutions with a GC×GC–FID instrument calls for a more specific detection, such as MS, which plays the role of a third dimension. In theory, quadrupole analyzers have a scanning rate (a few Herz) too slow to be hyphenated to the fast GC column of the second GC×GC dimension. The ideal detection should be achieved with a time-of-flight MS that allows detection frequencies of more than 50 Hz (18). Such instruments are still too expensive for a control quality objective. However, monitoring a single ion per compound may be sufficient for a quantitative analysis. In such a way, a sampling rate of 30 Hz can be achieved with a routine instrument.

This approach has been applied to re-evaluate the spiked SVB sample in which quantitation was unsuccessful using GC–MS and

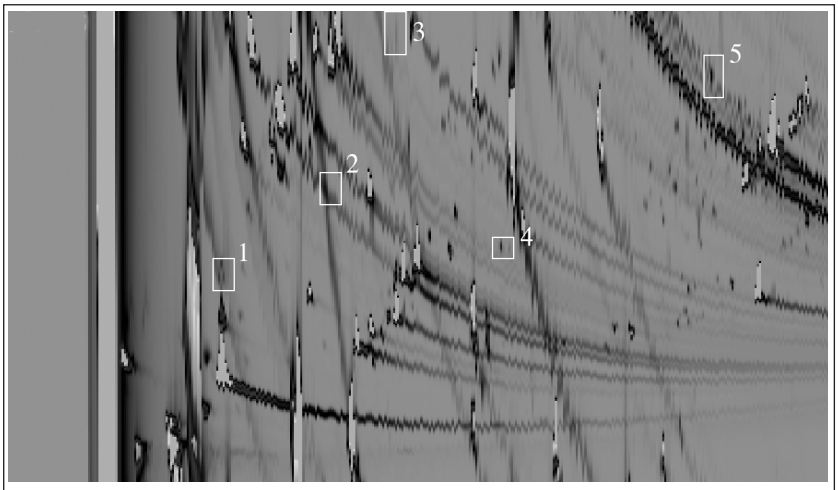

Figure 4. GC×GC–FID analysis of a fragrance concentrate (168 constituents) spiked with five target compounds at a level of 50 mg/L (2D chromatogram generated by GC-Image). (1) Linalool, (2) anisic alcohol, (3 and 3') eugenol, (4) α-isomethylionone, and (5) benzyl benzoate.

Table IV. GC–MS, GC×GC–FID and GC×GC–MS Quantitation of a Fragrance Concentrate* Spiked with Five Allergens at Individual Levels of 50 ppm

Column	GC–MS PDMS	GC×GC–FID PDMS×PEG	GC×GC–MS PDMS×OV225	GC×GC–MS PDMS×OV225
Data treament	MS data analysis (17)	GC data analysis + MathLab (17)	MS data analysis + spreadsheet (17)	GC-Image
Linalool	6296†	510†	57	41
Anisyl alcohol	782†	NQ	54	52
Eugenol	43	50	70	48
α-Isomethylionone	64	52	53	53
Benzyl benzoate	54	54	61	51
Mean	NA	NA	59	49
RSD	NA	NA	12	10

* SVB, 168 ingredients.
† Coelutions; NQ = not quantitatable; and NA = not applicable.

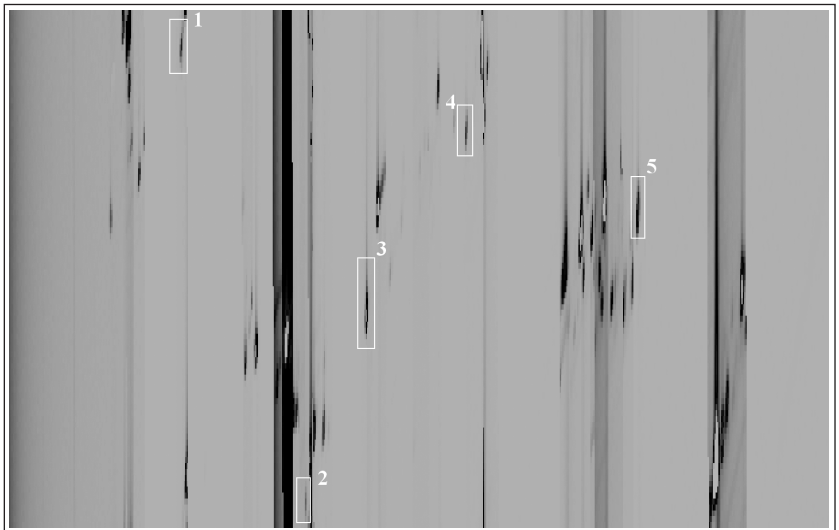

Figure 5. GC×GC–MS analysis of a fragrance concentrate (168 constituents) spiked with five target compounds at a level of 50 mg/L (2D chromatogram generated by GC-Image). (1) Linalool, (2) anisic alcohol, (3) eugenol, (4) α-isomethylionone, and (5) benzyl benzoate.

GC×GC–FID (Table IV) (17). A PDMS×PEG configuration led to a satisfactory result but polar compounds gave broad peaks in the polar second dimension (data not shown). Decreasing the polarity to that of a OV225-type column gave good evaluations of the allergen content (Table IV).

The dramatic improvement of the detection selectivity is exemplified by comparing 2D chromatograms of GC×GC–FID and GC×GC–MS analyses performed with the same sample (Figures 4 and 5). As the latter was achieved in SIM mode, only a few peaks remained visible, among them spiked allergens appeared as clear blobs well separated from their neighbors.

Improvement of the GC×GC–MS data treatment

The previous investigations were achieved by integrating GC peaks using the GC or GC–MS data analysis option of the instrument software. The integration results were the transferred into a spreadsheet to calculate the area sums from the different modulations of each target peak (peaks must be manually chosen before being summed by the spreadsheet program). This step was a major limitation to the use of GC×GC–q-MS because of the huge time consumption for the analyst. Therefore a software prototype was tested. This program, called GC-Image, has been developed by the University of Nebraska (19). It allows importation of data files in CSV format and calculation of blob volumes. 2D chromatograms of Figures 4 and 5 were generated using this program. Calibration curves of all target compounds were re-established, using the calculated blob volumes, from the previous GC×GC–MS analyses (17) and compared with those based on the transfer of integration results into a spreadsheet (Table V). Correlation coefficients were similar except for three compounds (hydroxycitronellal, amylcinnamic alcohol, and the second peak of farnesol). These differences seemed to be caused by the choice of integration parameters of these peaks. Using an MS software and spreadsheet, there was a "manual" intervention of the analyst who could refine integration parameters and "interpret" integration results (choice of the peaks belonging to the modulation of a same analyte). In contrast, GC-Image automatically collects the modulated peaks belonging to a same compound by using the same set of parameters for the whole acquisition. This could explain some differences between both data treatments, and the slightly greater dispersion of the correlation coefficients using GC-Image. However, the overall calibration quality was

Table V. Comparison of the Calibration Quality (R^2) Using the Blob Volume Determined by GC-Image and by the Area Sum of the Modulations (28 Compounds)

	Sum of areas (spreadsheet)	Blob volumes (GC-Image)
Hydroxycitronellal	0.9985	0.9701
Amylcinnamic alcohol	0.9728	0.9977
Farnesol II	0.9993	0.9395
Mean over all compounds	0.9979	0.9947
SD*	0.0051	0.0123
RSD†	0.51	1.24

* SD = standard deviation.
† RSD = relative standard deviation.

similar as shown by the average correlation coefficients (Table V).

Quantitative results of previously reported analyses of allergens were reprocessed using GC-Image (Table IV, last column). Surprisingly, these results were even better than using a combination of MS and spreadsheet softwares, as shown by the average amount (49 mg/L) close to the spiked amounts (50 mg/L) and the lower relative standard deviation. However, the main advantage was the dramatic shortening of the data treatment by a factor of 4–5 times.

Conclusion

This review shows that the quantitation of allergens has made significant progress over the past few years, from the initial GC–FID procedure up to the use of GC×GC–MS. Although the IFRA method represents the most reliable, readily accessible method for quantitating these substances, automated reporting procedures of commercial MS softwares sometimes face difficulties. In such circumstances, the strategy of the lowest amount from three ions may solve coelution problems, and the full-scan analysis allows detecting retention time shifts. The use of comprehensive multidimensional GC in combination with a quadrupole MS seems to overcome the quantitation of allergens in very complex fragrance concentrates. The time-consuming data treatment of this uncommon hyphenation can be shortened by an appropriate software. More work is needed to investigate both the applicability limit of the three-ion strategy, and the feasibility of speeding up the scan rate of quadrupole MS to provide analysts with a low-cost routine GC×GC–MS system.

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