Strategies to Analyze Suspected Allergens in Fragrances

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The 7th amendment of the European Cosmetics Directive (2003/15/EC) was published earlier this year.[1] Among other things it will, when enacted into national legislation, require manufacturers of cosmetics to indicate in their ingredient statements, the names of 24 chemically defined substances (T-1) and two natural extracts when they are present at concentrations exceeding 0.001 percent in cosmetics that are intended to remain on the skin, or 0.01 percent in those that are rinsed off the skin. As opposed to other labelling rules that relate only to intentionally added ingredients, the requirement to label these 26 purportedly allergenic materials will depend on their concentrations in the cosmetic products regardless of whether they have entered the product by direct addition or as constituents of essential oils, natural extracts or even as impurities in synthetic ingredients. Rules such as these can only be adhered to and policed if there exist reliable validated analytical methods for quantifying the presence of these substances.

List of the 24 suspected allergens[1] **T-1**

Name	CAS Reg. N°	Name	CAS Reg. N°
Amylcinnamic alcohol	[101-85-9]	Eugenol	[97-53-0]
Amylcinnamic aldehyde	[122-40-7]	Farnesol	[106-28-5]
Anisyl alcohol	[105-13-5]	Geraniol	[106-24-1]
Benzyl alcohol	[100-51-6]	Hexylcinnamic aldehyde	[101-86-0]
Benzyl benzoate	[120-51-4]	Hydroxycitronellal	[107-75-75]
Benzyl cinnamate	[103-41-3]	Isoeugenol	[97-54-1]
Benzyl salicylate	[118-58-1]	Butylphenyl methylpropional	[80-54-6]
Cinnamic alcohol	[104-54-1]	Limonene	[5989-27-5]
Cinnamic aldehyde	[104-55-2]	Linalool	[78-70-6]
Citral	[5392-40-5]	Hydroxyisohexyl-3-cyclohexene carboxaldehyde	[31906-04-4]
Citronellol	[106-22-9]	Methyl 2-octynoate	[111-12-6]
Coumarine	[91-64-5]	α-Isomethylionone	[127-51-5]

For this reason, the International Fragrance Association (IFRA) has set up a working group on analytical methods in order to develop analytical techniques that not only meet these requirements but are also accessible to the technical resources of most manufacturers in the industry. Efforts were made through this association and the European Flavour and Fragrance Association (EFFA) to ensure that the result of this collective effort could also be of benefit to others. In particular, a close collaboration with the European Centre for Standardization (CEN) has been made by one of the present authors (A. Chaintreau) on behalf of EFFA. It is hoped also that through these efforts, other analytical chemists such as those working for consumer protection groups, will be made aware of some of the potential errors that can be made when less reliable methods are used.

This paper aims to describe the analytical strategy that has been developed at Firmenich to monitor suspected allergens in fragrance concentrates. First the routine IFRA method will be briefly described, as it is intended to be the "common language" among fragrance companies, suppliers and customers. However, due to the extreme complexity of fragrance

mixtures, some cases may require more sophisticated tools to solve ambiguities. Two different non-routine approaches are presented: the former calls for more sophistication from the MS side (GC/MS with chemical ionization). The latter requires more sophistication on the chromatographic side, owing to comprehensive GC.

Routine GC/MS Analysis

Previous literature: Due to the huge number of constituents in fragrance concentrates, monitoring allergens using a conventional GC equipped with a flame-ionization detector (FID) is not conceivable. A paper by Rastogi proposes target compound identification via GC/MS under electron impact (EI) ionization and quantification with flame-ionization detection.[2] This obviously leads to the same risk of co-elutions as a simple GC/FID analysis. In a later report, a variant based the determination on ion traces extracted from a GC/MS analysis in scan mode.[3] However, scan acquisitions are known to yield less accurate results than selected ion monitoring (SIM).[4] Such a SIM approach was proposed by Ellendt using two ions to achieve the quantification.[5] Recoveries in the 98 percent to 106 percent range and a detection limit of 2 mg/L were claimed. However, under similar conditions, quantification by spiking experiments of a sample at a level of 20 mg/L with two ions in SIM mode was shown to be beyond of the method capability.[6]

IFRA method: To overcome result discrepancies between laboratories applying different analytical procedures, Firmenich, in collaboration with other ; of a unified method in the framework of IFRA.[6, 7] GC/quadrupole-MS instruments, which are common in the fragrance industry today, were the chosen method due to high selectivity and good quantitative performances. In this method, the quantification is performed in SIM mode. The chromatogram is divided into retention time windows; each of them successively monitors one or two target compounds. Three ions are used per compound: one for the quantification and the others (the "qualifiers") to confirm the peak identity. Because of the uncommon complexity of allergen analysis, experimental parameters are carefully optimized (as seen below).

GC conditions: The best separation of all target compounds was obtained for OV17-type columns; however, OV1-types were also suitable and were chosen due to their larger availability in QC laboratories.

Brominated internal standards were used due to their stability and characteristic ions. Isooctane and o-fluorotoluene were chosen as solvents because they did not promote the degradation of standards, unlike protic solvents, and were not prone to rapid evaporation from calibration solutions.

Evaluation of a fragrance ("proton") spiked with 50 mg/L of compounds reported in bold, using GC/MS-EI and a DB17, or GC/MS-CI and a DB1 column **T-2**

Name	GC/MS-EI Amount (mg/L)	Q	GC/MS-CI Amount (mg/L)
Phenylacetaldehyde	4.7	75	
Linalool	2.9	26	
Estragole	46.5	99	45.5
Citronellol	329.5	24	
Geraniol	45.1	99	64.6
Hydroxycitronellal	44.5	99	58.7
Methyl 2-nonynoate	42.7	98	46.2
Methyleugenol	47.7	98	33.1
Amyl cinnamic aldehyde	14.7	26	
Amyl cinnamic alcohol	442.15	80 [a]	
Farnesol	28.7	1	
Hexyl cinnamic aldehyde	6.7	66 [a]	
Benzyl benzoate	3.7	69 [a]	
Benzyl salicylate	47.1	77 [a]	

a: Absence checked in scan mode

The stock solution of the standard mix was found to be stable for one month in a freezer, or two months in the refrigerator if carbonyl compounds were separated from others.

The calibration appeared to be stable for only one week. Cleaning the injector weekly was also recommended because of the accumulation of low-boiling ingredients of fragrances.

To exemplify the capability of the method to be extended to other compounds used in fragrances, phenylacetaldehyde, estragole, methyl eugenol and methyl 2-nonynoate were included in the analyses shown in this paper. The mean recovery calculated from the spiking of three fragrances with 50 or 100 mg/L of various target compounds, was 100.5 percent, with a coefficient of variation of 16 percent (one example in T-2, GC/MS-EI column). Very importantly, calculating the "Q-value" from the target ion area and areas of two qualifier ions checked the identity of each peak. Depending on its value, possible co-elutions may also be suspected but not always solved.

From the information in T-2, it appears that 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 spite of the GC/MS selectivity, co-elutions 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 re-calibration of the instrument. This gives rise to time-consuming analyses for a single sample.

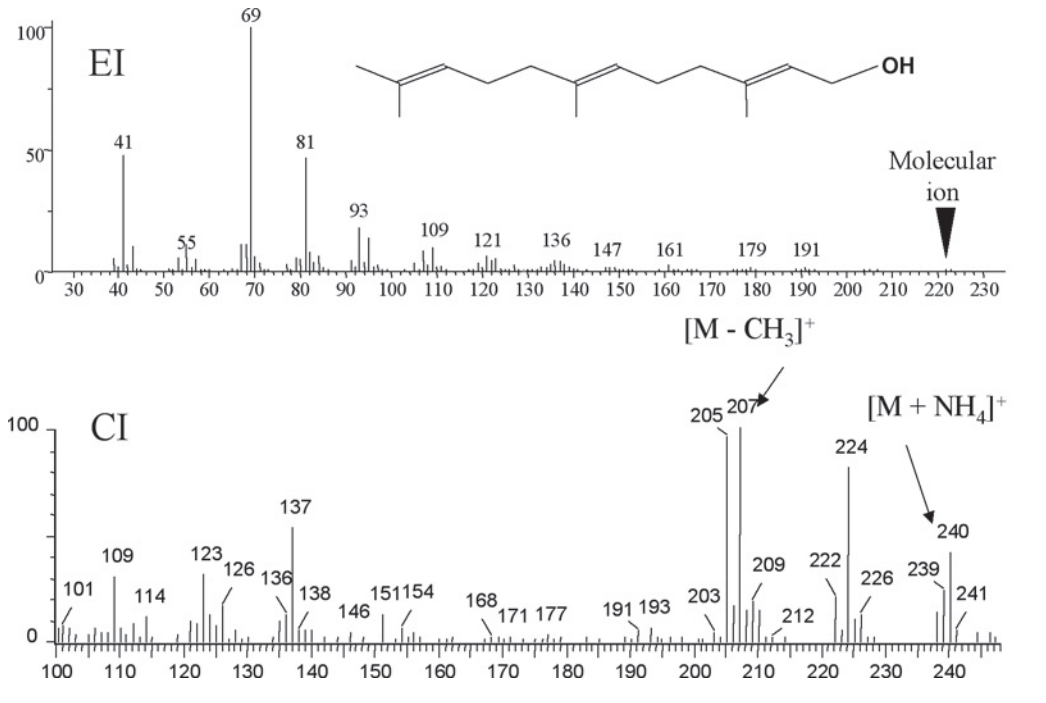
Firmenich's QC laboratories now routinely use the present GC/MS method to monitor allergens in fragrance concentrates. However, to overcome co-elution cases in a shorter way than by re-injecting the sample under different GC/conditions, alternative techniques were investigated.

MS-Based Alternative: GC/MS-CI

This new approach is based on the application of an ammonia chemical ionization (CI) GC/MS to produce the quasi-molecular ion of target compounds. The shortcoming of the above-described routine GC/MS method based on electron impact (EI) is that the mass spectra for some of the suspected allergens under investigation (especially terpenes) result in very common ions and lacks a characteristic molecular ion. This can lead to an overestimation for some of the allergens. The ammonia CI was developed to address the unresolved allergen quantifications of the routine method (e.g. T-3, first column). In comparison to the conventional EI ionization, CI ammonia is a gentle and soft ionization that provides the advantages of simpler cracking patterns, intense quasi-molecular ion and offers more selectivity for the detection of the suspected allergens. Because of its chemical selectivity, the ammonia reagent ion reacts with the allergens in a perfume matrix to give simple spectra such as ammonium adducts (1), or proton transfer reactions (2):

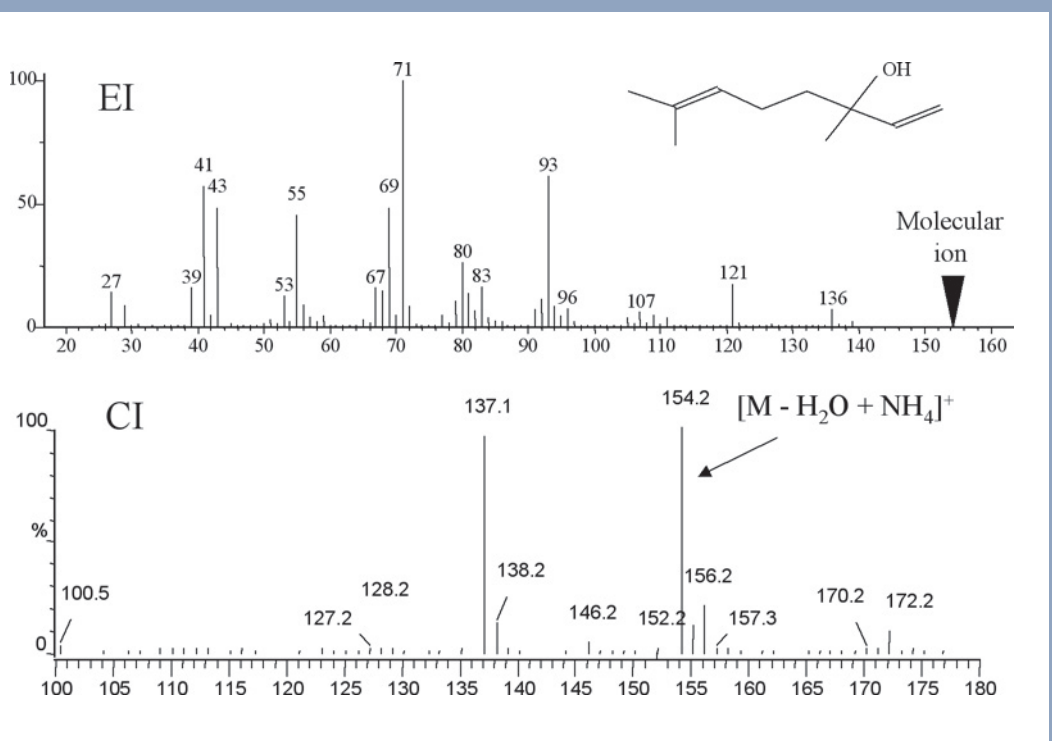
MS spectrum of farnesol under electron impact (EI) and ammonia chemical ionization conditions

F-1



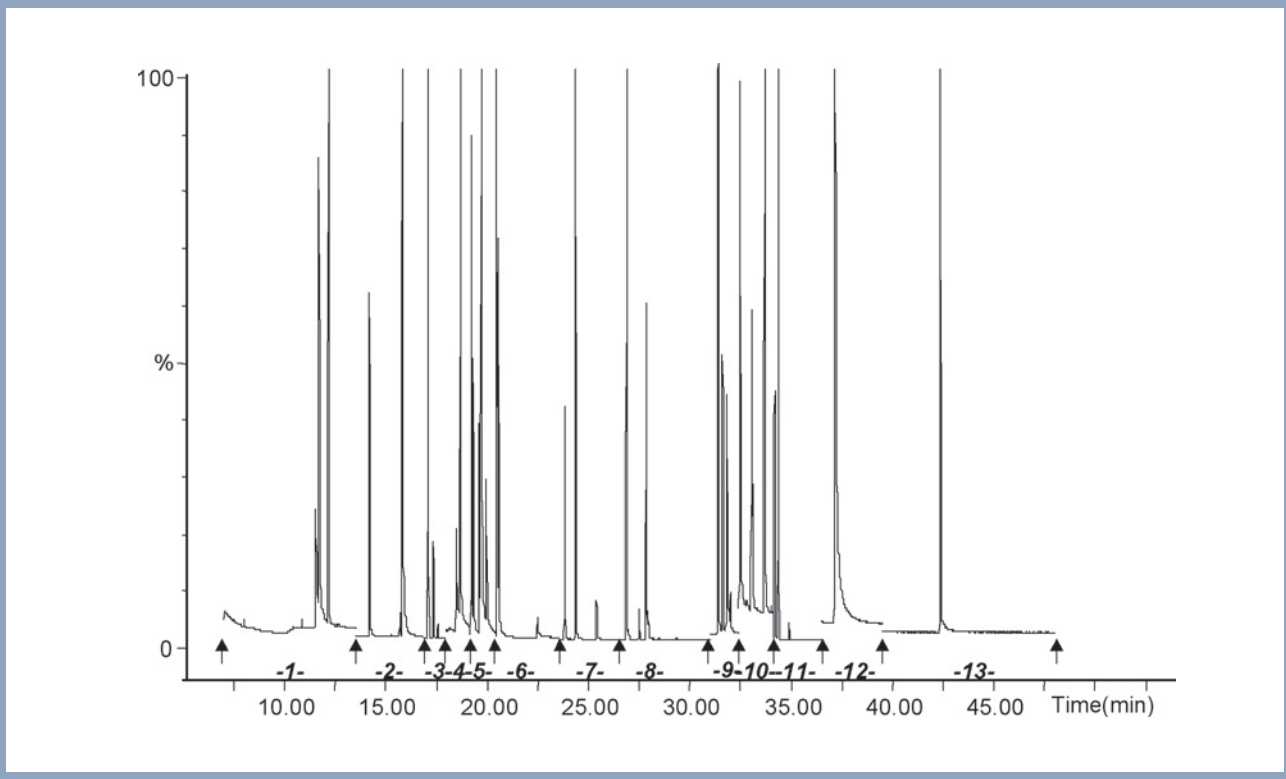
MS spectrum of linalool under electron impact (EI) and ammonia chemical ionization conditions

F-2



GC/MS-CI of the reference mixture with a DB1 column — the whole run is divided into 13 time-windows (limited by two arrows): 1. benzyl alcohol (11.53), phenylacetaldehyde (11.70), limonene (12.15); 2. linalool (14.18), iodoanisol (IS, 15.68), sulfolane IS, 15.82; 3. methyl 2-octynoate (17.09), estragole (17.34), OFN (IS, 17.58); 4. citronellol (18.47), neral (18.68); 5. cinnamaldehyde (19.21), geraniol (19.31), geranial (19.61), anisic alcohol (19.71), hydroxycitronellal (20.00); 6. methyl 2-nonynoate (20.50), cinnamic alcohol (20.56), eugenol (22.48); 7. methyleugenol (23.62), coumarine (24.36), isoeugenol (25.39); 8. α-isomethylionone (26.89), butylphenyl methylpropional (27.84); 9. amylcinnamaldehyde (31.40), hydroxyisohexyl-3-cyclohexene carboxaldehyde (31.62); 10. amylcinnamic alcohol (32.47), farnesol (33.05); 11. hexylcinnamaldehyde (34.21), benzylbenzoate (34.40); 12. benzyl salicylate (37.20); 13. benzyl cinnamate (42.36)

F-3



$NH_4^+ + Allergen \ddagger [Allergen + NH_4]^+$ (1)

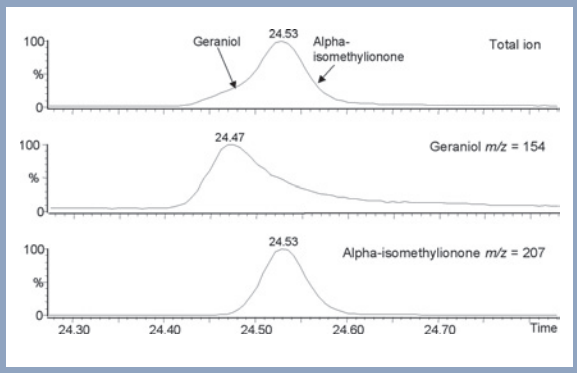
$NH_4^+ + Allergen \ddagger [Allergen + H]^+ + NH_3$ (2)

Ammonium adducts and proton transfer ions are preferentially observed with polar and basic compounds, respectively.[8-12] Non-polar and non-basic compounds, such as hydrocarbons, are poorly ionized. Because CI is a soft ionization, little fragmentation occurs, and intense peaks at high masses are obtained, which leads to a better sensitivity and selectivity than EI spectra. As an example, the EI spectrum of farnesol exhibits non-specific, low mass fragments ions that make its quantification difficult with the routine method, whereas with ammonia CI a typical set of ions is formed: $(M + NH_4)^+$, $(M + NH_4 - CH_4)^+$, $(M - CH_3)^+$. These ions enable the confirmation of the molecular mass and the classification and identification of this compound by its specific ions and intensity patterns (F-1). In the same way, linalool yields a quasi-molecular ion $(M + NH_4 - H_2O)^+$ as a base peak, whereas no molecular ion is detected in EI (F-2).

As linear calibration curves are observed ($r^2 >$ 0.998%, 20 to 500 mg/L), GC/MS-CI can be used as an alternative method to overcome co-elution problems occurring in GC/EI-MS. F-3 shows a chromatogram of the target compound mixture, where 13 times and selective mass windows have been defined. Each of them monitors two to 10 ions, corresponding to characteristic quasi-molecular ions of one to three compounds under NH_4^+ ionization.

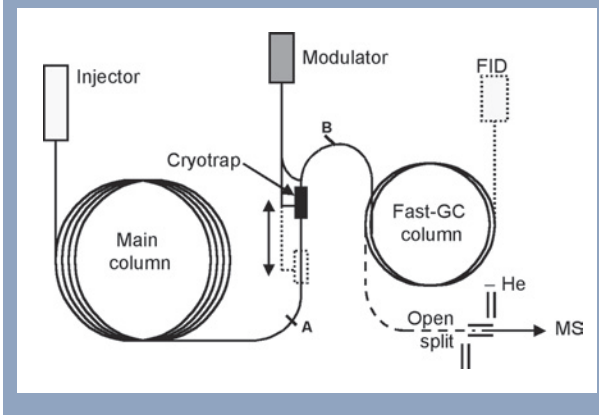
Co-elution of geraniol with α-isomethylionone with a Carbowax column (top), and peak resolution using ammonia chemical ionization: geraniol (middle, m/z = 154) and α-isomethylionone (bottom, m/z = 207)

F-4



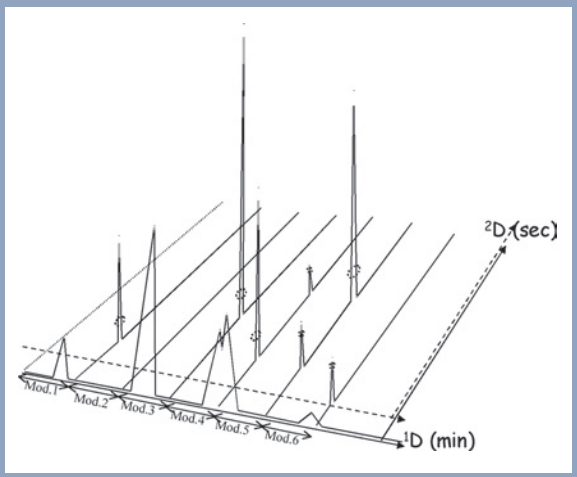
Scheme of a comprehensive GC based on a longitudinally modulated cryogenic system (LMCS), according to Marriott;[14] the second dimension is hyphenated either to an FID (short dotted line) or to an MS (long doted line)

F-5



Schematic 3D chromatogram generated by a comprehensive GC system; the chromatogram along the first axis (^{1}D) represents the trace that would be observed with a single-column GC — dotted axes and circles indicate what a — "contour plot" is (intersection of the 3D chromatogram with a plane above original axes)

F-6



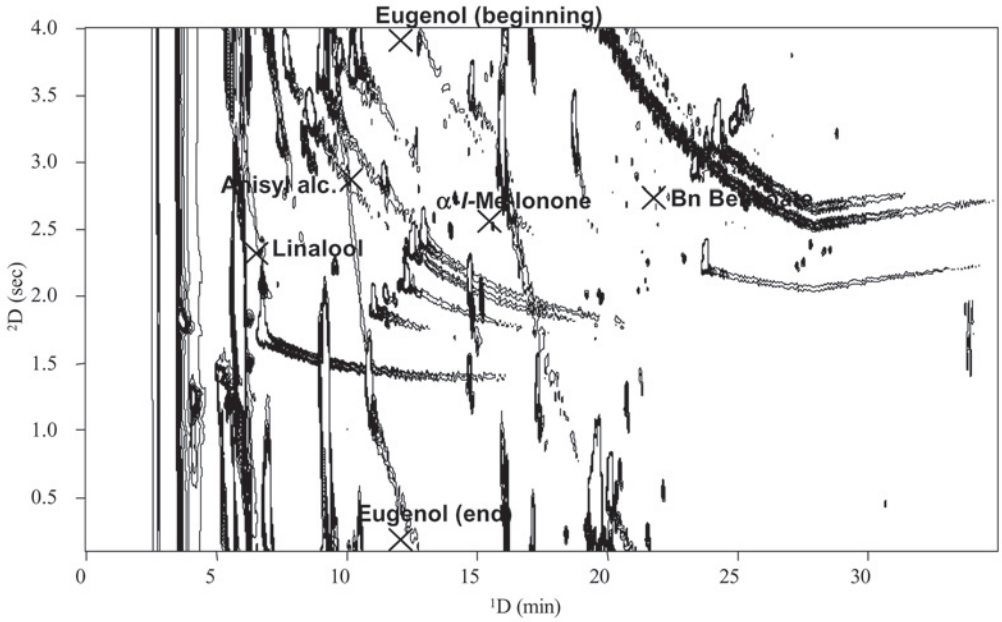
The quantification by CI is achieved by using quasi-molecular ions. The fragment ions are used as a confirmation tool. Therefore CI can more easily address the problems of the co-elution than the electron impact by using the selective mass chromatogram of each compound (F-4). The overlap of geraniol and α-isomethylionone peaks is resolved owing to the selectivity of ions 154 and 207 for geraniol and α-isomethylionone, respectively.

For simple fragrances, the quantitative perfor–mances of the GC/MS-CI method compares favorably to those of GC/MS-EI according an inter-laboratory test (T-2, last column: mean quantity over the five target compounds spiked at a 50 mg/L level: 49.6 mg/L, with a mean relative standard deviation of 25 percent). In addition, for very complex samples, its capability to provide a quantitative evaluation when the routine method fails is exemplified in T-3. A very complex allergen-free fragrance ("SVB") was spiked with five target compounds at a 50 mg/L concentration. Linalool and anisyl alcohol could not be estimated using an electron impact ionization due to the co-elution of other fragrance ingredient with isobaric ions. The GC/MS-CI method gives good quantification results with a satisfactory mean relative standard deviation (21 percent).

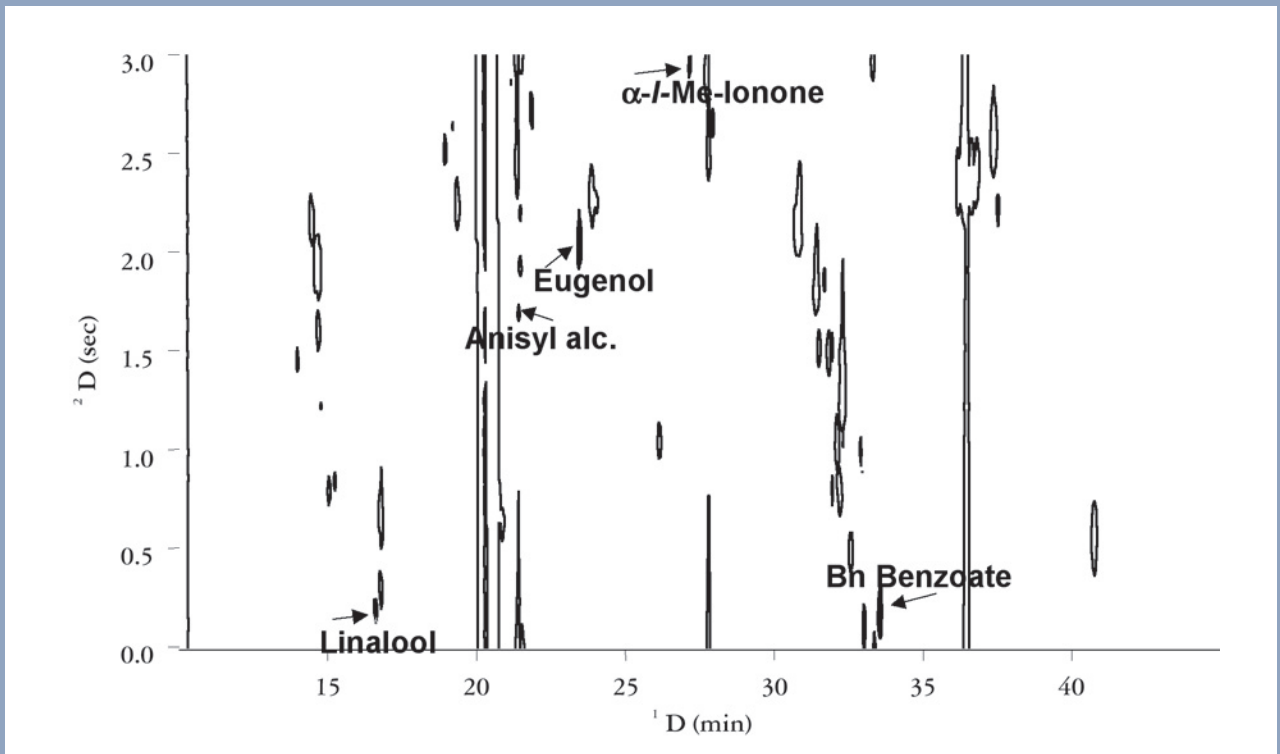
Comprehensive GC

The general GC limitation of flavor and fragrance analyses is the peak capacity of chromatographic columns compared to the great number of possible constituents. Comprehensive GC, a recent technique introduced by Phillips in 1991, seems to be able of a promising breakthrough.[13] Two columns are coupled in series such that all analytes eluting from the first are re-chromatographied in the second. The capillary portion between both columns is inserted into a modulator made of a moving cryotrap. When this latter is in the upper position (F-5, black-filled rectangle), peaks are retained in the cold zone for

Contour plot of the GC X GC/FID analysis of a 168-constituent fragrance spiked with five target compounds **F-7**



Contour plot of the GC X GC/MS analysis in SIM mode of a 168-constituents fragrance spiked with five target compounds **F-8**



GC/MS-EI, GC/MS-CI and GC X GC/MS quantification of a fragrance concentrate ("SVB") spiked with five compounds at a 50 ppm level **T-3**

Column Compound	GC/MS-EI DB1	GC/MS-CI DB1	GC x GC/FID DB1 x SPBWax	GC x GC/MS DB1 x DB225
Linalool	6296*	57	510*	57
Anisyl alcohol	782*	67	nq	54
Eugenol	43	39	50	70
α-Isomethylionone	64	45	52	53
Benzyl benzoate	54	62	54	61
Mean	na	54	na	59
RSD	na	21	na	12

*co-elutions; nq: not quantifiable; na: not applicable

typically 1 s to 5 s. Then the trap is pushed down (dotted rectangle) and back to its original position so that analytes are re-injected in the second fast-GC column and eluted within a few seconds. This modulation process is continuously repeated for the duration of the analysis and gives rise to the succession of short chromatograms along the second axis (F-6).

The principle and applications of comprehensive GC, also referred to as GC X GC, have been described in various reviews.[15, 16] To our knowledge, the present application to allergens represents its first use in the flavor and fragrance industry, excepting some papers on essential oil analysis made by public laboratories.[17, 18]

GC X GC/FID: Using a DB1- and a Carbowax-type column for the first and the second dimension, respectively, all target compounds were resolved without requiring a tedious optimization of GC conditions, in contrast to the development of the previous GC/MS method. As calibration curves were linear ($r^2 > 0.995$, 20-1000 mg/L), the technique was applicable to quantification.

If GC x GC/FID allows full separation of target compounds in simple fragrances, co-elutions still occur in complex mixtures.[19] As an example, the "SVB" fragrance concentrate (168 ingredients) was spiked with five target compounds. Two of them were not resolved and were thus quantified by comprehensive GC using a DB1 x Carbowax configuration (T-3). The same two compounds in the same fragrance were also not correctly quantified by GC/MS using the same column as the

first GC x GC dimension (DB1) (T-3). These difficulties are clearly illustrated in the corresponding 2D chromatogram (F-7), where tridimensional peaks are represented as contour plots according to their projection in the 2D space made of both retention time axes. The poor resolution of linalool from its neighbor is visible. Eugenol and anisic alcohol peaks do not appear using the threshold of the contour plot used. A lower threshold would result in a much more complex figure.

GC x GC/MS: As GC x GC/FID fails to solve co-elutions in complex mixtures, a more specific detection is required. A mass spectrometer can be used to provide the system with a third dimension. In spite of the low sampling rate of conventional quadrupole MS in regards to the fast elution of analytes from the second dimension, the detection rate gives satisfactory measurements of peak areas by monitoring a single ion for each of the target compounds.[20] This allows a good quantification of target compounds using a reasonable-cost instrument. Results obtained from a 168-ingredients fragrance analyzed with the four techniques are compared in T-3. Two of the five spiked compounds could not be evaluated by GC/MS or GC x GC/FID due to co-elutions with other constituents. In contrast, GC x GC/MS gives evaluations close to the expected value (50 mg/L).

The improvement is clearly illustrated by the GC x GC/MS contour plot (F-8). All spiked target compounds are visible and well separated from any other peak. Similarly to the use of SIM mode for mono-dimensional GC/MS analysis, monitoring a single ion greatly simplifies the 2D chromatogram.

Due to the lack of software dedicated to GC x GC/quadrupole-MS, the present method still requires some time investment from the analyst. Therefore its use remains limited to cases where the routine GC x GC/MS method fails.

Conclusion

The routine GC/MS method is efficient to monitor allergens in the great majority of samples. However, co-elutions sometimes occur and their resolution in MS-EI can become very time-consuming. This problem can be overcome by increasing the selectivity either of the MS detection owing to chemical ionization, or of the chromatographic selectivity using comprehensive GC. Both techniques, GC/MS-CI and GC x GC/MS, give similar quantitative results for the spiked complex fragrance concentrate used in this paper. However, the GC/MS-CI and GC x GC/MS are more sophisticated techniques requiring some more expertise from the operator side than GC/MS-EI, which limits their use for routine analysis.

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