THE INTERNATIONAL FRAGRANCE ASSOCIATION

ANALYTICAL WORKING GROUP

ANALYTICAL METHOD TO QUANTIFY 57 SUSPECTED ALLERGENS (AND ISOMERS) IN READY TO INJECT FRAGRANCE MATERIALS BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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List of Abbreviations

AWG	Analytical Working Group
C	Concentration
EC	European Commission
FID	Flame Ionisation Detection
GC	Gas Chromatography
GC-FID	Gas Chromatography with Flame Ionisation Detection
GC-MS	Gas Chromatography and Mass Spectrometry
IFRA	International Fragrance Association
IS_A	Internal Standard 1,4-Dibromobenzene
IS_B	Internal Standard 4,4'-Dibromobiphenyl
ISTD	Internal Standards
MS	Mass Spectrometry
MTBE	Methyl tert-butyl ether
MW	Molecular Weight
R	Resolution
RI	Retention Index
RN CAS	Registry Number from the Chemical Abstracts Service
R^2	Coefficient of determination
RT	Retention Time
RRF	Relative response Factor
RRF_{exp}	Experimental Relative response Factor
RRF_{pred}	Predicted Relative response Factor
SCAN	Full mass acquisition
SCCS	Scientific Committee on Consumer Safety
SIM	Single Ion Monitoring
S-ISTD	Stock solution of Internal Standard

1. Introduction

Regulation (EC) N°1223/2009, replacing Council Directive 76/768/EEC, relating to cosmetic products, regulates the obligation to inform consumers of the presence of 24 chemically-defined fragrance substances identified as potential allergens in cosmetic products. Following the publication of the Scientific Committee on Consumer Safety's document (SCCS/1459/11), it was proposed to extend that to 57 fragrance substances, some of them existing under several isomeric forms or as mixtures. This required the development of a new quantification method in response to the evolution of regulatory requirements.

The new analytical method has been developed using gas chromatography and mass spectrometry (GC-MS), to detect and to quantify the 57 fragrance substances and their relevant isomers at a concentration higher than 0.002% (20mg/kg) in ready to inject fragrance raw materials and oils. This level differs from the Legislation position of 0.001% (10mg/kg) in leave-on and 0.010% (100mg/kg) in rinse-off commercial products as the fragrance oil will only be used at a dosage of up to approximately 20% in the final commercial product. If the fragrance oil is to be used at 100% (i.e. as 'The Product') then the procedure should be followed as per a complex raw material and adapted to deal with the concentrations found.

The present document describes a working analytical method based on IFRA Analytical Working Group developments.

1.1. Scope

The present method allows the laboratory to identify and to quantify the volatile compounds which are suspected to be allergens in fragrance compositions and raw materials used in cosmetic products. The analysis is performed by GC-MS on matrix samples which are "ready to be injected" and which are compatible with gas chromatography.

The analytes covered by this procedure are based on the contents of Tables 13.1 and 13.2 in the SCCS Opinion document (reference) and as listed in the legislation proposed by the European Commission. The rationale behind the final choice of procedure analytes is given in the **Table 18** found in **Appendix J**.

2. Principle

This procedure has a calibration range from 0.002% (20mg/kg) to 0.25% (2500mg/kg) per analyte; beyond the upper concentration level the recommendation is that the sample should be diluted further, or that GC-FID (GC with Flame Ionisation Detection) is used preferably in combination with internal standard and response factors.

Following dilution of the matrix sample in an inert solvent, the suspected allergens are analysed by GC-MS in a total of 4 runs, using 2 sets of analytes, both injected on 2 separate columns of differing polarity.

Their identification and quantification is achieved through selected ion monitoring (SIM; SIM-SCAN or SCAN) mode via the relative abundance of 3 characteristic fragment ions. The calculation and use of the corresponding Q value or similar data evaluation factor can be applied and a 'Decisional Tree' for the final inspection and validation of the data by a trained and experienced analyst is described. An additional full-SCAN analysis is recommended to confirm the presence of the allergen in matrix samples for SIM only methodologies.

Their quantification is achieved in all modes by calibration using standard solutions and the internal standards 1,4-Dibromobenzene and 4,4-Dibromobiphenyl. The 'Decisional Tree' is employed to determine the final concentration taking into account the different concentration values obtained from analysis on both columns.

This Procedure is written as a methodology to be applied in a working laboratory. Particular attention must be paid by the user to the details contained in the Appendices and to specific comments ('Notes') in the text regarding the application of this methodology.

3. Reagents

3.1. Solvent

Methyl pivalate

RN CAS [598-98-1]

Purity ≥ 99%

Distillation to obtain a purity >99.9% is recommended to eliminate impurities, which are likely to interfere with the signal of analytes such as terpenes. If a commercial grade is used then that must be analysed and confirmed as not giving rise to interfering artefacts.

3.2. Reference samples (suspected allergens)

Initially, the purity of all standards should be measured by GC-FID (**Appendix A**) if not certified by the supplier. The precise RN CAS of the target analyte and the rationale behind this is given in **Table 18** in **Appendix J**.

3.2.1 **alpha Acetyl cedrene (principle isomer in Vertofix® referred to in RN CAS)**, RN CAS [32388-55-9]

Note: highly variable composition with at least twelve constituents of molecular weight equal to 246.

3.2.2 **Acetyl isoeugenol / Isoeugenyl acetate,** RN CAS [93-29-8]

3.2.3 **Amyl salicylate**, Pentyl salicylate RN CAS [2050-08-0]

Note: this can contain Isoamyl salicylate (N° RN CAS [87-20-7]), which should not be included in this assay.

3.2.4 **alpha Amyl cinnamaldehyde (Flosal®)**, RN CAS [122-40-7]

Note: 2 possible isomers (*E, Z*); only the E isomer (RN CAS [78605-96-6]) is quantified in this procedure.

3.2.5 **alpha Amylcinnamyl alcohol**, RN CAS [101-85-9]

Note: 2 possible isomers (*E, Z*); only the E isomer (RN CAS [184900-07-0]) is quantified in this procedure.

3.2.6 **Anethole,** RN CAS [4180-23-8],

Note: 2 possible isomers (*E, Z*); only the E isomer (RN CAS [4180-23-8]) is quantified in this procedure.

3.2.7 **Anise alcohol**, RN CAS [105-13-5]

Note: Only the Para- (4-methoxy) isomer (RN CAS [105-13-5]) is quantified in this procedure.

3.2.8 **Benzaldehyde**, RN CAS [100-52-7]

3.2.9 **Benzyl alcohol**, RN CAS [100-51-6]

3.2.10 **Benzyl benzoate**, RN CAS [120-51-4]

3.2.11 **Benzyl cinnamate**, RN CAS [103-41-3]

Note: only the E isomer (RN CAS [103-41-3]) is quantified in this procedure.

3.2.12 **Benzyl salicylate**, RN CAS [118-58-1]

3.2.13 **Camphor**, RN CAS [76-22-2, 464-49-3]

Note: RN CAS [464-49-3} relates to the (d-) optical isomer of camphor and is not selectively analysed in this procedure. Only the racemic form RN CAS [76-22-2] is quantified.

3.2.14 **Carvone**, RN CAS [99-49-0, 6485-40-1, 2244-16-8]

Note: RN CAS [6485-40-1] and [2244-16-8] relate to the optical isomers (l-) and (d-) Carvone respectfully and are not selectively analysed in this procedure. Only the racemic form RN CAS [99-49-0] is quantified.

3.2.15 **beta Caryophyllene**, RN CAS [87-44-5]

3.2.16 **Cinnamaldehyde**, RN CAS [104-55-2]

Note: 2 possible isomers (*E, Z*), only the E isomer (RN CAS [104-55-2]) is quantified in this procedure.

3.2.17 **Cinnamyl alcohol**, RN CAS [104-54-1]

Note: 2 possible isomers (E, Z), only the E isomer (RN CAS [104-54-1]) is quantified in this procedure.

3.2.18 **Citral,** RN CAS [5392-40-5]

Note: both Neral (*Z* isomer, RN CAS [106-26-3] and Geranial (*E* isomer, RN CAS [141-27-5]) are quantified.

3.2.19 **Citronellol**, RN CAS [106-22-9; 1117-61-9; 7540-51-4]

Note: RN CAS [1117-61-9] and [7540-51-4] are the optically active 3R (d-) and 3S (l-) forms of Citronellol. These are not selectively analysed in this procedure. Only the racemic form RN CAS [106-22-9] is quantified.

3.2.20 **Coumarin**, RN CAS [91-64-5]

3.2.21 **beta Damascenone** (Rose Ketone-4), RN CAS [23696-85-7]

3.2.22 **alpha Damascone**, RN CAS [43052-87-5]

Note: 2 possible isomers (*E, Z*); only the *E* major isomer (92 to 99%) should be quantified.

3.2.23 **beta (E) Damascone**, RN CAS [23726-91-2]

3.2.24 **delta Damascone** (Rose Ketone-3), RN CAS [57378-68-4]

Note: three possible isomers (trans/trans, cis/trans, trans/cis) but only the major isomer (trans/trans, up to 90%) should be quantified.

3.2.25 **Dimethylbenzylcarbinyl acetate (DMBCA)**, RN CAS [151-05-3]

3.2.26 **Ebanol,** RN CAS [67801-20-1]

Note: the 2 isomers (E, Z) are quantified. The procedure measures two components (Ebanol 1; Ebanol 2) which comprise unresolvable, multiple diastereoisomeric pairs. There are no RN CAS which adequately describe these pairs. The sum of these two isomers is used for the final declared values of Ebanol.

3.2.27 **Eugenol**, RN CAS [97-53-0]

3.2.28 **Eugenyl acetate**, RN CAS [93-28-7]

3.2.29 **Farnesol**, RN CAS [4602-84-0]

Note: 4 possible isomers: (*E,E*) isomer is (RN CAS [106-28-5]) and the (*Z,E*) isomer is (RN CAS [3790-71-4]). RN CAS [4602-84-0] refers to generic Farnesol. This comprises four isomers of which the primary (*E,E*)-Farnesol (RN CAS [106-28-5]) is used as the calibration standard for the other isomers if found to be present.

3.2.30 **Galaxolide (Hexamethylindanopyran**), RN CAS [1222-05-5]

Note: Only the two main isomers are quantified.

3.2.31 **Geraniol**, RN CAS [106-24-1]

3.2.32 **Geranyl acetate**, RN CAS [105-87-3]

3.2.33 **Hexadecanolactone / Dihydroambrettolide**, RN CAS [109-29-5]

3.2.34 **alpha Hexylcinnamaldehyde (Jasmonal®)**, RN CAS [101-86-0]

Note: 2 isomers at least; only the E isomer (RN CAS [165184-98-5]) is quantified in this procedure.

3.2.35 **Hydroxycitronellal**, RN CAS [107-75-5]

3.2.36 Tetramethylacetyloctahydronaphthalene (ISO E® Super)

Note: this consists of several isomers including the alpha, (RN CAS [68155-66-8]); the major isomer, beta (RN CAS [54464-57-2]); the gamma (RN CAS [68155-67-9]) and a minor (RN CAS [54464-59-4]). Only beta (RN CAS [54464-57-2] main isomer), alpha (RN CAS [68155-66-8]) and gamma (RN CAS [68155-67-9]) isomers are quantified to represent the total Tetramethylacetyloctahydronaphthalene (ISO E® Super) concentration.

3.2.37 Isoeugenol, RN CAS [97-54-1]

Note: 2 possible isomers (*E, Z*), only (*E*)-Isoeugenol should be analysed.

3.2.38 alpha Isomethylionone, RN CAS [127-51-5]

Note: can contain beta Isomethylionone (RN CAS [79-89-0]), alpha Methylionone (RN CAS [7779-30-8]), beta Methylionone (RN CAS [127-43-5]), pseudo Isomethylionone (RN CAS [1117-41-5]). FYI – Other grades may contain other isomers including the major isomer. These constituents should not be assayed.

3.2.39 Butylphenyl methylpropional (Lilial®), RN CAS [80-54-6]

3.2.40 Limonene, RN CAS [5989-27-5]

Note: RN CAS [5989-27-5] relates to d-limonene and is not resolvable from the l-form by this procedure. Therefore only the racemate RN CAS [138-86-3] is analysed.

3.2.41 Linalool, RN CAS [78-70-6]

3.2.42 Linalyl acetate, RN CAS [115-95-7]

3.2.43 Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®), RN CAS [31906-04-4]

Note: contains Hydroxyisohexyl 4-cyclohexene carboxaldehyde (RN CAS [51414-25-6]) (noted HICC minor), which should be quantified.

3.2.44 Trimethyl-benzenepropanol (Majantol®), RN CAS [103694-68-4]

3.2.45 Menthol, RN CAS [1490-04-6, 89-78-1, 2216-51-5]

Note: RN CAS [1490-04-6] and [2216-51-5] relate to the optical isomers of menthol. These are not resolvable in this procedure and therefore only the racemic form (RN CAS [89-78-1]) is quantified.

3.2.46 Methyl salicylate, RN CAS [119-36-8]

3.2.47 Methyl-2-octynoate (Folione®), RN CAS [111-12-6]

3.2.48 alpha Pinene, RN CAS [80-56-8]

3.2.49 beta Pinene, RN CAS [127-91-3]

3.2.50 **3-Propylidene phthalide**, RN CAS [17369-59-4] ((*E*)-isomer: RN CAS [56014-72-3] and (*Z*)- isomer: RN CAS [94704-89-9])

Note: the 2 isomers (*E:Z*) should be quantified (isomers ratio near 1:6).

3.2.51 **Salicylaldehyde**, RN CAS [90-02-8]

3.2.52 **Santalol**, RN CAS [11031-45-1]

Note: the 2 isomers, alpha Santalol (RN CAS [115-71-9]) and beta Santalol (RN CAS [77-42-9]), should both be assayed.

3.2.53 **Sclareol**, RN CAS [515-03-7]

3.2.54 **alpha Terpinene**, RN CAS [99-86-5]

3.2.55 **alpha Terpineol**, RN CAS [98-55-5] for alpha Terpineol, the primary isomer to be quantified by the procedure.

3.2.55a for gamma Terpineol RN CAS [586-81-2];

3.2.55b for *cis*-beta Terpineol RN CAS [138-87-4];

3.2.55c for *trans*-beta Terpineol RN CAS [7299-41-4]

3.2.56 **Terpinolene**, RN CAS [586-62-9]

3.2.57 **Vanillin**, RN CAS [121-33-5]

3.3. Internal standards (ISTD)

3.3.1. 1,4-Dibromobenzene (IS_A)

RN CAS [106-37-6], purity ≥ 98%.

3.3.2. 4,4'-Dibromobiphenyl (IS_B)

RN CAS [92-86-4], purity ≥ 98%.

4. Equipment

4.1. A gas chromatograph equipped with flame ionization detector (GC-FID)

This apparatus is only used to determine the purity of reference samples intended to be used for calibration purposes and the ISTDs before quantitative analysis if required, or for the quantification of high concentration analytes (> 2.5% as found in essential oils for example). It is recommended to perform the purity study using the procedure described in **Appendix A**.

A GC-FID method is available[2] for the measurement of analytes at concentrations > 2.5%. Note that this method does not implicitly cover all the allergens mentioned in this procedure: however, the principle covered in that method can be applied to all the analytes contained within the proposed legislation and this procedure.

4.2. A gas chromatograph coupled to a mass spectrometer (GC-MS)

This apparatus is used for quantitative analysis, to check for the presence and measure the concentration of the suspected allergens. The system must be able to comply with the following requirements:

4.2.1. GC-MS System

a. Equipped with a split/splitless injector fitted with a glass liner maintained at 250°C (or at a temperature in accordance with the manufacturer's recommendations).
b. Equipped with electronic control of carrier gas pressures and/or flows.
c. It is recommended that an autosampler, fitted with a syringe of suitable size, be used for the injection of the calibration and sample solutions.
d. The glass injection liner must be inert with an interior volume compatible with the expansion volume of the dilution solvent.
e. The carrier gas is helium with a constant flow.
f. The MS source temperature should be set at 250°C (a compromise to protect fragile components and to limit condensation of the heavy components in the transfer line).
g. The MS quadrupole temperature is set at 150°C.
h. Two capillary columns of different phase types are to be used for quantitative analysis.
i. Mass spectrometer tuning and the levels of air and water should be checked weekly, as well as the injector cleanliness to maintain analytical performance.
j. The sensitivity of the mass spectrometer should also be optimized through maximising the detector signal to noise ratio. It is recommended that this is done by increasing the detector (electron multiplier) voltage according to the instrument capabilities and manufacturers recommendations (typically + 200V above the default autotune setting).

4.2.2. Other analytical equipment

All weighing of reference samples and internal standards must be carried out on an analytical balance with 0.0001g readability.

4.3. Capillary columns for GC

The two capillary columns described in **Table 1** below are those recommended for this analysis. The use of any alternative columns requires the User to undertake a full evaluation of the impact on peak resolution to be investigated prior to use. The User must be satisfied that all target peaks can be adequately resolved and that no interference of the SIM ions used for quantification takes place.

Note 1: Whilst the retention times and SIM windows have been specified for the columns mentioned, these are for guidance only and the user must verify all compound retention times and the associated SIM windows for their own installations.

Note 2: The column lengths are those used for the initial development and validation by the AWG. In practice, additional separation of the target analyte may be achieved by moving to a longer column (50 or 60m). If such columns are used, then it is the User's responsibility to validate the performance of that column type/length in the context of this method (resolution; SIM windows; possible coelutions) and validate that length for the analytes(s) being investigated.

Note 3: Where there is a shared ion in the same retention time window and components are not fully resolved, then a change in column flow *may* achieve better separation. Analysts should be aware that this situation has been reported in the case of alpha Damascone and beta Damascenone.

Table 1 - Recommended GC Column Phase/Types

Phase	Dimensions (Length × internal diameter × thickness)	Recommended oven temperature program	Mean Resolution $\bar{R}$
100% Polydimethylsiloxane (OV1 phase)	30m × 0.25mm × 0.25µm	80°C for 4 mins, then 15°C/min to 105°C for 2 min, then 4°C/min to 150°C, then 10°C/min to 270°C	SIM method 1 1.43
			SIM method 2 1.40
50% Phenylmethylpolysiloxane 50% Polydimethylsiloxane (OV17 phase)	30m × 0.25mm × 0.25µm	80°C for 1 min, then 10°C/min to 135°C for 2 min, then 3°C/min to 170°C for 1 min, then 10°C/min to 280°C	SIM method 1 1.40
			SIM method 2 1.42

- For both columns, the carrier gas is helium with a constant flow set at 1.2ml/min (velocity approximately 40cm/sec).
- The recommended injection volume for calibration solutions and prepared samples is 1µl in split mode, with a recommended split ratio of 20:1 at a temperature of 250°C. The same split ratio must be used for calibration and sample analysis.

It is the responsibility of the analyst to monitor the condition of the columns in use. In practice, the determination of the mean resolution ($\overline{\mathbf{R}}$) is useful to survey the separation performance of the selected columns during all their period of use. The calculations needed to undertake this are detailed in **Appendix B**.

Note 4: It has been noted also that deviations from linearity can occur for some analytes at the higher calibration levels. This effect may be due to "peak skewness" at these concentrations. Peak symmetry limits between 0.8 and 1.2 have been proposed (see H.1.1).

5. Mass Spectrometer Acquisition Conditions

5.1. General Information

This method is based on the use of single quadrupole mass spectrometer instruments in the electron ionisation mode with conventional 70eV ionisation.

Use the mass spectrometer in accordance with the manufacturer's instructions and with respect to good mass spectrometry practices.

Each time an instrument tune is performed, the calibration may be modified and **therefore the calibration for the allergens must be re-established.**

5.2. SIM Mode; SIM-SCAN and SCAN only Modes – Establishment of Retention Times and SIM Windows

Operating in SCAN mode, inject one of the standard solutions (High to mid calibration concentrations for example) to determine the retention times and suitable SIM windows for each analyte.

For each column, the User will create two SIM methods (SIM1 and SIM2) with which to analyse the 66 potentially allergenic substances in two sets of analytes.

Typical SIM ions (I1, I2 and I3) and their associated Internal Standard are detailed in Tables 7-9 as given in Appendix C.

5.3. SIM Window – Set up and Criteria

Typical fragment ions to be used for this analysis are listed in **Tables 7 and 9** (for OV1-type column) and **Table 8** (for OV17-type column) **(see Appendix C for details)** in SIM mode to detect and to quantify suspected allergens in samples.

Note 5: Alternative ions are available as listed in the tables in Appendix C. Table 9 offers alternative quantifier and qualifier ions on OV1 type columns. The choice of the SIM ions must be validated by the individual User for effectiveness in their own application and alternative ions may give better performance.

The given m/z mass values are rounded to nominal mass units, but it is recommended to use the accurate mass values determined in full scan mode. In rare cases, the selected ions for one given allergen on a polar column can be different from the selected ions for a non-polar column.

The choice of internal standard to be assigned for quantification of each analyte is also provided in **Appendix C (Tables 7-9).**

As far as possible, each SIM window should not include more than 6 ions. Nevertheless, in the regions of the chromatogram where the elution of the analytes are close to one another, the SIM windows *can* contain up to 9 or more ions. In each case, the dwell time should be chosen to have a minimum of 3 scan cycles/sec, and/or at least 10 to 15 scans per chromatographic peak for accurate integration.

The width of the SIM window must also take into account peak shape (including potential peak tailing) and possible elution delay to facilitate further peak integration. The same acquisition time must be applied for all allergens ions in the same SIM window.

Note 6: The maximum number of SIM ions per window should be determined by the user for their own MS systems. This will depend on the age of the system and that manufacturer's own recommended limits. Exceeding the recommended number of ions per window will lead to poor performance of the Procedure and the generation of poor results.

The SIM-SCAN method is developed by selecting this mode in the mass spectrometer software using the SIM ion and SIM window data. Recent versions of MS software enable the analyst to adjust the relative time the instrument spends collecting SIM or SCAN data. It is recommended that the analyst ensures that sufficient data points are available to fully describe the peak for correct integration.

Examples of SIM windows for the two sets of analytes and the two columns are given in **Appendix D (Tables 10-13).**

Note 7: ***These are for illustrative purposes only and must be determined and validated by each user for their own particular system.***

If a SCAN only acquisition is made then the appropriate 3 ions may be extracted for each analyte and the resulting peak area obtained by integration using the system's software. The fixed SIM acquisition window for the analytes can be adjusted to take into account the elution delay, which can be observed when an abundant component of the fragrance oil elutes from the capillary GC column just before an allergen.

6. Stock and Sample solutions - preparation and storage

6.1. General information

6.1.1. Choice of the solvent

Methyl pivalate is suitable for the preparation of stock and calibration solutions as well as the dilution of samples prior to analysis as it has been proven to satisfy the following requirements:

1. Inertness to components and allergens in fragrance oils
2. Low volatility to ensure solution's stability and concentration,
3. Expansion volume compatible with the interior volume of the injector insert.

Its purity should be controlled before use; its distillation is recommended to eliminate impurities, which are likely to interfere with the signal of analytes such as terpenes.

Alternative solvents are available (e.g. Methyl tert-butyl ether – MTBE). If these are used for this method, then the Operator must undertake sufficient evaluation to verify that the solvent contains no constituents that would otherwise interfere with the analytes in this method.

The use of any other solvent requires the operator to perform preliminary tests to demonstrate, in particular, inertness towards the analytes under investigation and for its purity to be determined. A highly volatile solvent should not be used for the preparation of stock solutions (for example, acetone; diethyl ether). In addition, the use of solvents possessing hydroxyl or carbonyl functions (for example, methanol; ethanol; acetone etc.) is not recommended in order to avoid degradation and/or reaction with the analytes under investigation (e.g. through the formation of acetal derivatives etc.).

6.1.2. Miscellaneous

The same solvent must be used to prepare the calibration solutions, the system blank and to dilute samples. A 10-fold dilution of samples in the solvent is recommended prior to the analysis to limit quantification error related to matrix effects (See **section 7**).

If the GC-MS system has been left 'idle' for any length of time, material can build up in the injection system and create interference with the analysis. It is therefore recommended that an initial blank solvent run be undertaken to clean the inlet of any residues prior to the start of the calibration/analysis sequence.

Note 8: Adequate blank values should be obtained before analysis commences. No analyte should be detected in the blank analysis above 75% of the lower calibration level. Blank corrections are not applied to this Procedure. A blank analysis is used within the analytical sequence to determine carry-over or contamination of the system. If analyte concentrations of > 75% of the lower level of calibration are seen in these samples, then the inlet system requires maintenance and the sequence from the last known 'good blank' is to be repeated.

6.2. Preparation of stock solutions from reference samples

6.2.1. General information

The preparation of the Calibration Stock Solutions and the subsequent Calibration Solutions can be made from two sources – from the single reference materials themselves (after purity determination/confirmation), or from commercially obtained Calibration Solutions of known constituent concentrations.

The following description covers making the calibration solutions from commercial Calibration Solutions of known concentration. **If the User wishes to follow the procedure for making these solutions from single reference materials, the procedure for this can be found in Appendix G.**

6.3. Preparation from Commercial Stock Standard Solutions

6.3.1. Stock Solutions of Internal Standards (1g/kg equivalent) (S-ISTD)

Prepare a stock solution (**S-ISTD**) containing 1g of 1,4-dibromobenzene (3.3.1) and 1g4,4'-dibromobiphenyl (3.3.2) made up to 1kg with methyl pivalate. This solution can be prepared as follows:

- Prepare a solution ($ISTD_0$) by weighing accurately 0.1g of each compound (3.3.1 and 3.3.2) in a suitable flask and make up to 100g with methyl pivalate.
- Note the weight of each compound and the final weight of solvent used, to determine the final solution concentration of each internal standard.

6.3.2. Calibration Solutions

The calibration solutions are prepared from two sets of 'stock' solutions, A1 and A2, containing the analytes at a nominal concentration of 10g/kg. The final calibration solutions are arrived at by a dilution of these solutions (or a further derivative of these; A3 and A4 – see below) to a known weight with methyl pivalate.

Note 9: For Commercial Standards of alternative concentrations, the dilution volumes used may need to be adjusted to arrive at the final concentrations as shown in Table 2 below.

To avoid dispensing very small volumes for the lower calibration concentrations from the stock solutions A1 and A2, if required, two further solutions **A3** and **A4** can be prepared using the following schemes (**Tables 2-3**).

These two sets of stock solutions (A1 and A2) and (A3 and A4), may be stored in darkness in a freezer at a temperature lower than -18°C for a maximum of one month. Ensure they are adequately stoppered to minimize any potential evaporation of the solvent.

Solution A3

Table 2 - Preparation volume for Solution A3

Primary Solution (10g/kg)	Volume of Primary Solution	Final Volume of Diluted Solution (A3)	Final Concentration of Allergens (mg/kg)
A1	10ml	1000ml	100

Solution A4

Table 3 - Preparation volume for Solution A4

Primary Solution (10g/kg)	Volume of Primary Solution	Final Volume of Diluted Solution (A4)	Final Concentration of Allergens (mg/kg)
A2	10ml	1000ml	100

6.3.3. Calibration solutions

Prepare the calibration solutions **C1, C2, C3, C4, C5** by diluting the two allergen primary solutions, **A1** and **A2** to 10ml with methyl pivalate after the addition of the internal standard solution, (**S-ISTD**), according to the following instructions, and referring to the dilution scheme in **Table 4**. **Record the weight of the individual combined solutions.**

Similarly, prepare the calibration solutions **C6, C7, C8, C9** by diluting solutions **A3** and **A4** to 10ml with methyl pivalate after the addition of the internal standard solution, (**S-ISTD**), according to the dilution scheme in **Table 4. The weight of the individual combined solutions must be recorded.**

6.3.3.1. Calibration Solution Dilution Process

To create the Calibration Solutions C1 through C9, the following process should be adopted:

- For Solution C1: Dispense 0.25ml of Solution A1 and 0.25ml of solution A2 into a 10ml tared volumetric flask, noting the weights of A1 and A2 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C2: Dispense 0.20ml of Solution A1 and 0.20ml of solution A2 into a 10ml tared volumetric flask, noting the weights of A1 and A2 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C3: Dispense 0.15ml of Solution A1 and 0.15ml of solution A2 into a 10ml tared volumetric flask, noting the weights of A1 and A2 taken at each step.

Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

Note the final weight.

- For Solution C4: Dispense 0.10ml of Solution A1 and 0.10ml of solution A2 into a 10ml tared volumetric flask, noting the weights of A1 and A2 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C5: Dispense 0.05ml of Solution A1 and 0.05ml of solution A2 into a 10ml tared volumetric flask, noting the weights of A1 and A2 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

For Solutions C6 to C9, the solutions A3 and A4 should be used to avoid potential errors in dispensing small amounts of A1 and A2:

- For Solution C6: Dispense 2.5ml of **Solution A3** and 2.5ml of **solution A4** into a 10ml tared volumetric flask, noting the weights of A3 and A4 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C7: Dispense 1.0ml of **Solution A3** and 1.0ml of **solution A4** into a 10ml tared volumetric flask, noting the weights of A3 and A4 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C8: Dispense 0.5ml of **Solution A3** and 0.5ml of **solution A4** into a 10ml tared volumetric flask, noting the weights of A3 and A4 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

- For Solution C9: Dispense 0.2ml of **Solution A3** and 0.2ml of **solution A4** into a 10ml tared volumetric flask, noting the weights of A3 and A4 taken at each step.

  Add 1ml of the Internal Standard solution and make up to the 10ml mark with solvent.

  Note the final weight.

These instructions can be summarized and found in **Table 4**.

Note 10: Any changes to the dilution scheme *must* result in the final calibration concentrations as given in Table 4 below.

Table 4 - Individual Allergen Calibration Standard Solution concentrations

Internal Standards concentration at 100mg/kg

	Allergens Concentration (mg/kg)	Volume of Allergen Solution (ml)	Volume of Allergen Solution (ml)	Volume of Internal Standard Solution added (ml)	Concentration of Internal Standards (mg/kg)	Final Volume of Calibration Solution (ml)
Primary Solutions Used		**A1 (10g/kg)**	**A2 (10g/kg**	**(1g/kg)**		
C1	250	0.250	0.250	1.000	100	10
C2	200	0.200	0.200	1.000	100	10
C3	150	0.150	0.150	1.000	100	10
C4	100	0.100	0.100	1.000	100	10
C5	50	0.050	0.050	1.000	100	10
Secondary Solutions		**A3 (0.1g/kg)**	**A4 (0.1g/kg)**	**(1g/kg)**		
C6	25	2.500	2.500	1.000	100	10
C7	10	1.000	1.000	1.000	100	10
C8	5	0.500	0.500	1.000	100	10
C9	2	0.200	0.200	1.000	100	10

General notes and information:

- In the absence of stability data, freshly prepare these solutions and do not store. Do not use these calibration solutions after 48 hours.
- **Note 11: This procedure results in one combined calibration solution that contains both primary solutions A1 and A2, or A3 and A4, together with the internal standards and which is used to construct each calibration level.**
- The potentially allergenic substances (57 fragrance substances and relevant isomers) are analysed as two sets of analytes (by 2 SIM methods or 2 SIM-SCAN methods) on each of the two capillary columns of different polarity, which corresponds to a total of 4 different analytical runs.
- It is recommended to run the calibration solutions from the lowest concentration to the highest concentration to avoid potential carry over influencing the calibration.

6.3.3.2. Plotting of a Calibration Curve

Inject each Calibration solution (C1 through C9) on the two separate GC-MS columns according to the SIM or SIM-SCAN methods.

For each allergen, each ion in **Tables 7-8 (Appendix C)** (e.g. **I1**) is used in turn as the quantifier ion, with the remaining two ions (e.g. **I2** and **I3**) being used as the qualifier ions.

One calibration per column type is to be constructed, which results in six curves per allergen component.

The calibration curve is built by plotting the area ratio between the analyte and the internal standard as a function of their concentration ratio.

The recommended calibration curve to apply is:

Quadratic Fit

Quadratic Fit is recommended and that a force through zero may be applied, but this requires the User to validate this as applicable to their system as this may mask issues with system cleanliness at low concentrations.

In addition, a weighting of 1/concentration of the calibration point may also be applied, though this is optional."

These settings have been arrived at through the application of the method to test mixes during the method development phase.

A more detailed explanation of the calibration procedure can be found in **Appendix H**.

7. Sample Analysis

As described in 7.1, a final 10X dilution of the sample to be analysed is required which includes the addition of the Internal Standards. It is important that the final concentration of the Internal Standards in the sample to be analysed are the same as those in the calibration standards.

Thus, prior to this dilution step, the analyst must ensure that the concentration of any analyte present must not exceed 2500mg/kg in either the preliminary solution or in the sample as presented, before this final dilution step. Above the level of 2500mg/kg, the concentration of the analyte in the final solution will exceed the working range of the method.

Therefore, the analyst must use their discretion in the preparation of the solution prior to the final step of dilution and addition of the internal standards. Any dilution regime can be applied (e.g. 10X; 100X; 200X etc.), providing the concentration of the analyte is reduced to below the 2500mg/kg level. It has been observed that preliminary sample dilutions greater than 250X may lead to problems of reproducibility; however, it is the analyst's responsibility to assess the overall acceptability of this if dilutions greater than 250X of the sample are contemplated.

If higher dilutions are not practicable and the analyst is confident that the results are not compromised by coelutions, for example, then a similar method to this but using GC-FID may be considered[2,4].

7.1. Sample Preparation for Analysis

- Accurately weigh (to the nearest mg) a sample amount, *w* (typically 1g), in a 10ml volumetric flask.
- Add 1.0ml of the internal standard solution (**S-ISTD**). The final concentration of internal standard added should be the same as that used in the calibration solutions.
- Adjust to the 10ml mark with methyl pivalate (or the same dilution solvent as that used to prepare calibration solutions).
- Accurately weigh the obtained solution to calculate the concentration (m_{DIL}). Note the final weight of this solution.
- Homogenize and transfer an aliquot of the prepared sample into a GC vial for GC-MS analysis.

7.2. GC-MS Analysis – Sequence

For the GC-MS analysis sequence, each vial, comprising the calibration solutions, the diluted samples, a calibration check solution or the blank, must be injected at least once under the given analytical conditions (e.g. each column type, SIM, SIM-SCAN or SCAN methods).

Note 13: The calibration check solution is used to ensure that the calibration has not drifted during the analytical sequence. The values from this analysis should ideally be no more than +/- 20% deviation from the expected concentration of each allergen at that solution concentration, or within the performance ranges defined by the laboratory. If results are found to be outside of these limits then the calibration sequence should be rerun, a new calibration established and the previous sequence repeated.

See Note 8 (section 6.1.2) above for blank value assessments and remedial action/impact if found to be outside of laboratory limits.

7.3. GC-MS Analysis - SAMPLE

Inject the diluted sample on the GC-MS using the same conditions as used for the calibration standards and on each of the two chromatographic columns, using the corresponding acquisition methods (SIM; SIM-SCAN; SCAN).

This should be performed within a 24 hour window following sample preparation.

Following the GC-MS analysis of the samples, check the concentration of found analytes, which must be within the calibration range.

If the declared concentration for an analyte is found to be outside of the calibration range, a greater dilution of the sample must be carried out before subsequent GC-MS re-analysis. If this is found to be the case, then a fresh sample must be prepared from the original sample with a greater dilution factor, i.e. 1g in 100ml or equivalent.

Note 14: It is important to note that over-dilution of the sample can lead to discrimination of higher M. Wt. constituents in the injection process and, therefore, will lead to skewed results. If a sample dilution of greater than 250-fold is required it is recommended that the analysis for that constituent is performed by GC-FID.

8. Data Validation and Treatment

There are three approaches to data validation: the use of Q values generated by some vendors' software as part of the analytical report, the use of corresponding SIM data to compare ion ratios or the use of scan data for ion ratios which is independent of the quantitation process. The Q value and SIM data is derived from peak area measurements whereas scan data provides direct ion abundances from which the ratios are calculated.

8.1. Examination of 'Q' Values.

The reported results from the GC-MS analysis using SIM can be initially evaluated by monitoring the 'Q' value. This gives an assessment of the validity of the value reported by comparing the relative area ratios for each of the three ions monitored for that component.

The calculation of the 'Q' factor is a standard calculation for some instrument vendors, but can also be calculated for those systems that do not have this ability.

The Q value is calculated from the peak area of the 3 different ions I1, I2 and I3, as follows:

$$Q = 100 - \frac{\sum_{i=1}^{i=n}(100\times|r_i - r_i'^1|)(ln[100r_i+1])}{21.3\times\sum_{i=1}^{i=n} r_i}^2$$

Where:
n is the number of qualifier ions for each analyte,
r_i is the reference area ratio and
r_i' is the experimental area ratio.

The identity of the suspected allergen is confirmed if one (at least) of the 6 Q values is higher than or equal to 90.

If the Q value is zero, the concentration corresponding to this value should not be used.

It is recommended to determine the reference area ratio from the injection of a standard solution.

Relative intensity of characteristic ions in SIM.

Alternatively, the positive identification of an allergen may be validated by considering the relative abundances of its characteristic ions from SIM area data.

The presence of the analyte is confirmed if the abundance of the three ions, on one of the two capillary columns, satisfies the criteria specified in **Appendix I.2 and I.3.**

Note 15: The table below may not be valid for SIM data, as the quantification ion chosen may not be the base peak in the spectrum of the component and the area of the base peak using SIM in those circumstances will be unknown.

8.2. Relative Ion Intensity using Scan Data

To identify an allergen, compare the relative abundances of ions in the sample to those obtained from a standard solution both generated in full scan mode. The maximum allowable tolerances for the relative ion intensities (expressed in percentage of intensity of the most abundant ion i.e. the base peak) are given below in **Table 5**.

Table 5 - Maximum allowable tolerances for the relative ion intensities (expressed in percentage of intensity of the most abundant ion i.e. the base peak)

Relative intensity	Threshold values
> 50%	± 10%
> 20% to 50%	± 15%
> 10% to 20%	± 20%
≤ 10%	± 50%

Using any of the approaches, it is recommended to determine the reference abundances of ions from the injection of a standard solution at a concentration in the mid-range of the calibration curve using the same analytical parameters.

The presence of the analyte is confirmed if the abundance of the three ions, on one of the two capillary columns, satisfies the criteria specified in **Appendix I.2 and I.3.**

8.3. Data Verification Scheme and reporting of Final Concentration

The choice of the final reported concentration should be performed in accordance with the requirements of the decisional tree as given in **Appendix F.** These steps are to be used to validate a positive result by allowing a structured evaluation of the data from all sources to be undertaken.

8.4. GC-MS Scan Mode Verification

A full SCAN mode is required to elucidate the presence of peaks that are thought to have moved out of their usual SIM window or been hidden by interfering peaks. The recommended scanning mass range (m/z) is from 35 to 350 Da.

8.5. Mass spectrum evaluation in SCAN mode

In case of questionable results, confirm or refute the results by studying sample chromatograms and analyte mass spectra after additional sample analysis in full scan mode. This approach could only be considered for relatively high concentrations of allergens as the SCAN mode is typically less sensitive than the SIM mode.

9. Assay report

The test report shall include at least the information required in the European standard EN 16274 [1].

10. References:

[1] “Méthode d’analyse des allergènes “, NF EN 16274, Octobre 2012

[2] “Rapid GC-FID quantification technique without authentic samples using predicted response factors” E. Tissot et al., Flavour Fragr. J. 2012, 27, 290–296

[3] “COMMISSION DECISION of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C(2002) 3044); Section 2.3.3.2 Mass Spectrometric Detection; L.221.16, 2002”

[4] “Application of gas-liquid chromatography to essential oils: XXII Determination of the 16 alleged skin sensitizers in essential oils” M.J. Milchard, R. Clery, L.Gates, F, Judge, N. Moss, D. A. Moyler, A. Sherlock, B. Starr, J. Webb, E.J. Newman., Perfumer and Flavorist, 2012, 37, 48-52.

APPENDIX A: Determination of calibrant and reference sample purity

A.1. General information

The use of GC-FID (Gas Chromatography with Flame Ionisation Detection) is recommended for the determination of the purity of a single reference calibration compound when no certified material is available.

The calibration solutions A1 and A2 can be derived from either commercially available standard solutions (in which case, this process can be avoided and the purities and weights applied from the supplier's information), or by making the standards within the users own facility, in which case this process should be used to estimate the constituent purity. In addition, it should be noted that purer materials can also be arrived at through alternative techniques including (but not limited to):

- Column Chromatography
- Distillation
- Fractionation
- Recrystallisation, etc.

The relative peak area (expressed as % area) of a volatile molecule measured by GC-FID is usually used to determine its purity in a mixture. This approach is exact if the two following conditions are fulfilled:

i. The mixture is only composed of volatile components that can be detected by GC,
ii. The relative response factor (RRF) of each component in the mixture, if differing one to another, is applied to deduce the quantity of each analyte from its chromatographic signal.

Accordingly, the purity of each reference sample used for quantitative analysis can only be determined after verification of the absence of non-volatile constituents in the sample. When more than 90% of the compounds are volatiles, standard purity can be estimated as follows:

i. If all the compounds exhibit the same molecular formula, their purity is given by their respective % FID area (with a full integration of all the detected peaks),
ii. If the compounds exhibit different molecular formulas, their purity can be estimated based on the difference between theoretical and observed RRF.

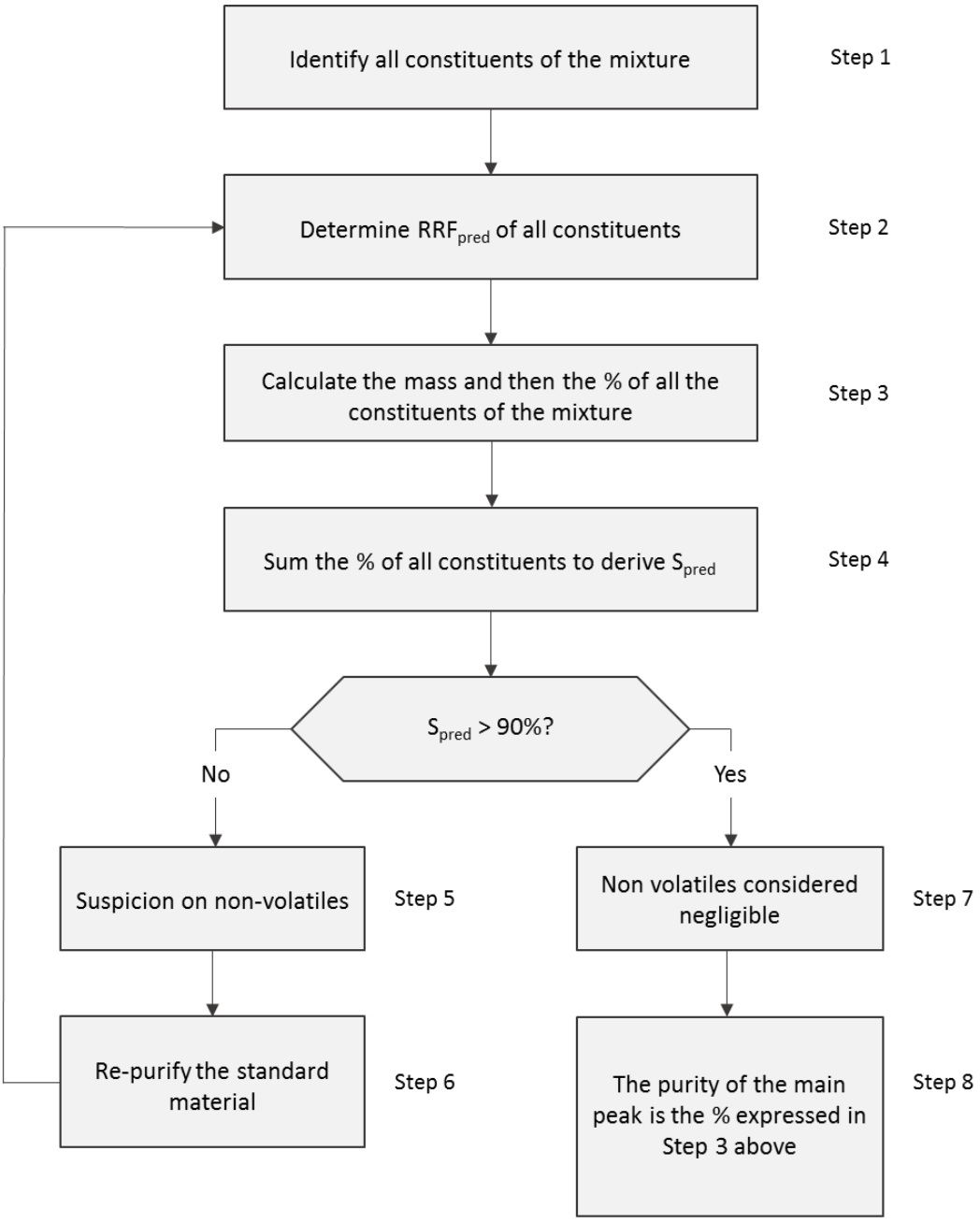
The different steps involved in this evaluation are:

i. The characterization of all the volatile constituents by GC-MS (**section A.2**),
ii. The estimation of the % of volatile compounds compared to non-volatile ones using RRF values,
iii. The estimation of allergen purity by:

    a. Measurement of individual % FID areas by GC-FID if all the compounds have the same formula.
    b. Comparison of predicted and calculated RRF values if the compounds do not have the same formula.

The general procedure is detailed in the following parts and can be summarized as follows (**Figure 1**):

Figure 1- General Procedure if impurities have differing molecular formula



Note: instead of calculating the % in Step 3, all the masses can be summed.
If this sum represents less than 90% of the total then there is a suspicion of non-volatiles in the sample.

(RRF = relative response factor)

A.2. Full characterisation of the volatile constituents

A.2.1 Identification of the individual constituents of the reference samples by GC-MS

Introduce into the GC-MS a 0.5 % w/w solution of an individual reference sample in the dilution solvent.

Confirm the identity of each molecule utilizing mass spectral data, retention index (RI), scientific literature, certificates of analysis from suppliers, etc. - Step 1 of the general procedure.

i. To identify the different isomers of interest

ii. To identify all the other constituents present in the mixture to obtain their exact molecular formula and the number of benzene rings (as RRF is directly related to the empirical formula of a compound, to distinguish related constituents (or impurities) of the same formula from those of different formula).

A.2.2. Calculation of predicted Relative Response Factor (RRF_{pred})

For each volatile constituent identified in **section A.2.1**, calculate the predicted RRF value according to the following formula (Step 2 of the general procedure).

For each molecule i, calculate predicted RRF using the following formula [5]:

a) $$RRF_{i\,pred} = {MW_i}/{(MRF_i \times MW_{IS})}$$

b) $$MRF_i = -0.071 + 8.57 \times 0.0001 \times \Delta H_{comb\ i} + 0.127 \times n_{Benz}$$

$$\Delta H_{comb} = 11.06 + (103{,}57 \times n_C) + (21.85 \times n_H) - (48.18 \times n_O) + (7.46 \times n_N) + (74.67 \times n_S) - (23.57 \times n_F) - (27.44 \times n_{Cl}) - (11.9 \times n_{Br}) - (2.04 \times n_I))$$

Where:
MW_i is the molar mass of the analyte i;
MW_{IS} is the molar mass of the internal standard (methyl-octanoate);
MRF_i is the molar response factor of analyte i;
$\Delta H_{comb\ i}$ is the combustion enthalpy of analyte i;
n_{Benz} is number of benzenic rings;
n_C, n_H, n_O, n_N, n_S, n_F, n_{Cl}, n_{Br}, n_I is the number of carbon, hydrogen, oxygen, nitrogen, sulphur, fluorine, chlorine, bromine, iodine atoms.

Note 16: This method allows the calculation of predicted RRF with the use of methyl octanoate as internal standard. If another internal standard is used, the predicted RRF might be corrected by the RRF of the new standard compared to methyl octanoate.

Note 17: This model of RRF prediction is applicable for any GC-FID system with a split/splitless standard injector maintained at 250°C and equipped with a tubular liner using a split ratio between 50:1 and 100:1. Cup-splitter liners and PTV injectors may not be used for this approach, in particular for less volatile compounds. FID temperature should be set at 250°C, with 40ml/min hydrogen, 450ml/min air, and a make-up of 30ml/min nitrogen. Both bonded polar and non-polar, or semi-polar columns can be used.

Be aware that changing injector temperature, split ratio, detector temperature is not suitable for the RRF prediction especially for the high-boiling compounds[6].

Polar column phases such as polyethylene glycol are also suitable, but the RRF of high-boiling compounds may also be modified.

Note 18: in the case of unknown compounds in the mixture:

- **The predicted RRF of a very similar compound can be used.**
- **A RRF equal to unity can be used as an ultimate solution if no clue about identification is found.**

A.2.3. Calculation of the amount of volatile constituents in the individual reference samples

- Inject the GC-FID working solution, containing the individual reference sample and IS (Methyl-octanoate, > 99% purity), all at 0.5% w/w in the dilution solvent.
- For each molecule i, detected in the reference sample, estimate the mass in the sample using the following formula (step 3 of the general procedure):

$$mass_{\ i} = RRF_{i\ pred} \times \frac{area_i \times mass_{IS}}{area_{IS}}$$

With:
$RRF_{i\ pred}$ *the predicted RRF as calculated in A.2.2.,*
$mass_{IS}$ *is the mass of internal standard involved in the sample preparation prior to injection,*
$area_i$ *is the FID area of compound I,*
$area_{IS}$ *is the area of the internal standard.*

- Estimate the amount of volatile constituents using the following formula:

$$\%\ of\ volatile\ constituents = \frac{\sum_{i=1}^{n} mass\ i}{mass\ of\ the\ reference\ sample}\ X\ 100$$

With:
n the total number of constituents i,
mass i is the mass calculated from predicted RRF,
mass of the reference sample is the mass of the reference sample involved in the sample preparation prior to injection.

If % of volatile constituents ≥ 90%: The fraction of non-volatile compounds in the reference sample is negligible, i.e. all the constituents of the reference sample can be detected by GC-FID (steps 7-8 of the general procedure).

If % of volatile constituents < 90%: non-volatile compounds are present in the reference sample but are not detectable using GC. Accordingly, purity calculations based on FID % area are inappropriate. In this case, it is recommended to re-purify the reference sample to eliminate non-volatiles components and to re-do the analysis (steps 5-6 of the general procedure).

A.3. Determination of purity (%) from RRF or % FID area

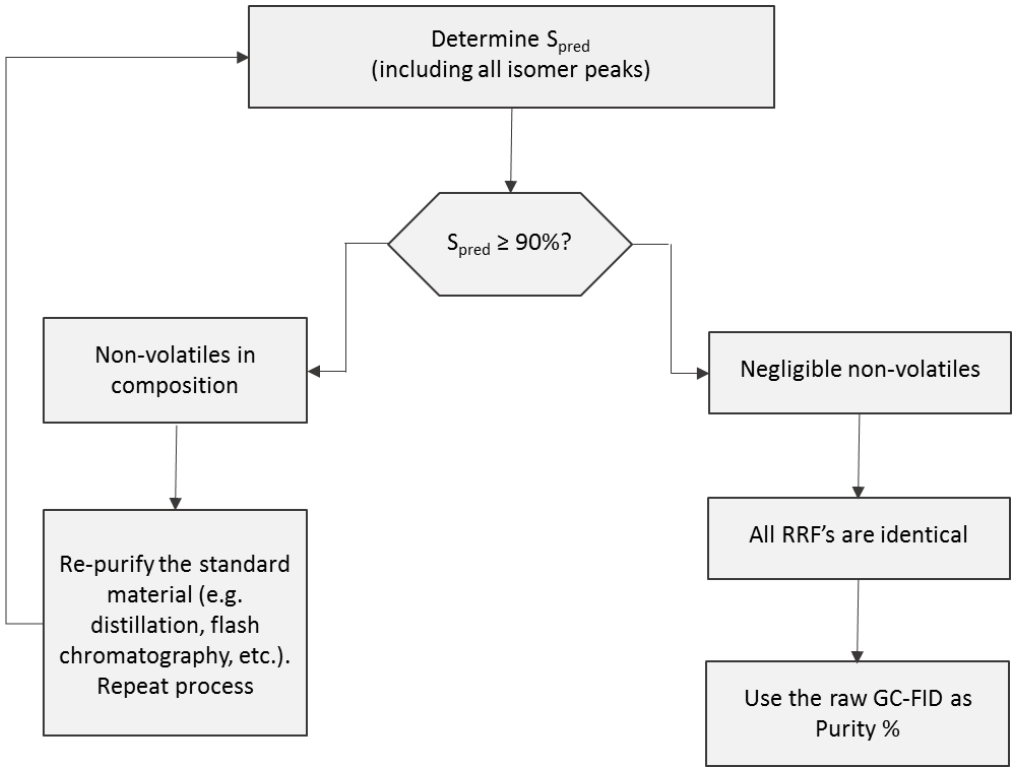
Depending on the nature of the volatile constituents in the reference sample, the purity can be determined directly using % FID areas or by comparison of predicted and experimental RRF (step 8 of the general procedure).

Details are provided hereafter and schematized in the purity decisional tree below.

1st case: all constituents of the mixture have the same formula as determined in section A.2.1.

In that situation, the following simplified procedure can be applied (**Figure 2**):

Figure 2 - Simplified Procedure if all impurities have the same molecular formula



Calculate purity of the molecule of interest using % FID area.

Integrate all the FID peaks except the internal standard if present and the peaks coming from the solvent and calculate the allergen(s) purity as follows:

$$S_{pred}(\%) = 100 \times {}^{FID\ area\ i}/_{FID\ area_{tot}}$$

Where:

S_{pred}: suspected standard purity,

FID area$_i$ and FID area$_{tot}$ are respectively allergen peak area and the sum of all peak areas measured in GC-FID.

Note 19: S_{pred} % can also be calculated as defined in the 2nd case; both ways of calculating will give the same result.

2nd case: all constituents of the mixture do not have the same formula as determined in part A.2.1.

In this case, all the constituents do not have the same RRF. Their relative amounts in the mixture have therefore, to be calculated after correction of individual experimental RRF (RRF_{exp}) with individual predicted RRF_{pred}.

- From the GC-FID working solution injected in **section A.2.3**, for each suspected allergen i, the calculation of $RRF_{i\ exp}$ is achieved using the following equation:

$$RRF_{i\ exp} = {(C_i \times area_{IS})}/{(C_{IS} \times area_i)}$$

Where:
C_i and C_{IS} are respectively allergen and internal standard concentration in the working solution, $area_{IS}$ and $area_i$ are respectively internal standard and allergen peak area measured by GC-FID. Concentrations can be replaced by the masses here.

Note 20: The number of solutions required to cover all the allergens is determined by the resolution achievable on the analytical columns used. Ideally, there should be no co-elution between allergens in these working solutions when determining RRF. The number of solutions analysed is the responsibility of the User to determine.

- For a suspected allergen molecule i, calculate RRF suspected allergen purity as follows:

$$S_{pred}\ (\%) = 100\ \times {RRF_{\mathrm{i\ pred}}}/{RRF_{i\ exp}}$$

Where:
S_{pred}: suspected standard purity,
RRF_{pred} : predicted RRF,
RRF_{exp}: experimental RRF.

A.4. References – Appendix A

[5] “Quantification in Gas Chromatography: Prediction of Flame Ionization Detector Response factors from Combustion Enthalpies and Molecular Structure”, de Saint Laumer et al, *Anal. Chem.* **2010,** *82,* 6457–6462.

[6] “Quantitation in gas chromatography: usual practices and performances of a response factor database”, Cicchetti et al. Flavour Fragr. J. 2008; 23: 450–459).

APPENDIX B: GC CAPILLARY COLUMN PARAMETERS

Table 6 - GC column parameters

Phase	Dimensions (Length × internal diameter × thickness)	Recommended oven temperature program	Mean Resolution $\bar{R}$ *
100% Polydimethylsiloxane (OV1 phase)	30m × 0.25mm × 0.25µm	80°C for 4 mins, then 15°C/min to 105°C for 2 min, then 4°C/min to 150°C, then 10°C/min to 270°C	SIM method 1 1.43
			SIM method 2 1.40
50% Phenylmethylpolysiloxane 50% Polydimethylsiloxane (OV17 phase)	30m × 0.25mm × 0.25µm	80°C for 1 min, then 10°C/min to 135°C for 2 min, then 3°C/min to 170°C for 1 min, then 10°C/min to 280°C	SIM method 1 1.40
			SIM method 2 1.42

In both cases, the carrier gas is helium with a constant flow set at 1.2ml/min (velocity near 40cm/s).

Alternative phase types and column lengths may be used for this method; however, it is the responsibility of the user to validate the separation capability and appropriateness for use of the chosen column in this application before being used to generate data. This procedure and its performance is based on the above column types and conditions

*To evaluate the separation performance of the columns, the resolution $\bar{R}$ of all neighbouring peaks and the mean resolution are calculated as follows:

$$\bar{R} = 1.18\left[\prod_{i=n}^{i=n-1}\left(\frac{t_{R,i+1} - t_{R,i}}{w_{h,i} + w_{h,i+1}}\right)\right]^{\frac{1}{n-1}}$$

Where:
$t_{R,i}$ is the retention time of the i^{th} peak,
$w_{h,i}$ is the half peak width of the i^{th} peak
and n is the peaks number on the chromatogram.

The separating efficiency of a given chromatographic column is considered as suitable if the resolution $\bar{R}$ is higher than 1 ($\bar{R} > 1$) and/or the mean resolution is higher than 1.3 (> 1.3).

APPENDIX C: SIM IONS FOR SIM; SIM-SCAN AND SCAN USE

Table 7 - SIM ions (m/z) of allergens listed in the elution order on OV1-type column, corresponding SIM methods (1 or 2) and internal standard (IS_A or IS_B) used for quantification

	OV1-type column					
	Identification	SIM Method	I1 (Da)	I2 (Da)	I3 (Da)	Internal Standard
3.2.8	Benzaldehyde	1	106	105	51	IS_A
3.2.48	alpha Pinene	2	93	92	91	
3.2.49	beta Pinene	2	93	92	91	
3.2.9	Benzyl alcohol	1	79	108	107	
3.2.54	alpha Terpinene	2	136	79	121	
3.2.51	Salicylaldehyde	1	122	121	65	
3.2.40	Limonene	2	68	93	67	
3.2.56	Terpinolene	2	93	136	121	
3.2.41	Linalool	2	93	71	80	
3.2.13	Camphor	1	95	108	81	
3.2.55b	cis-beta Terpineol	2	71	93	136	
3.2.55c	trans-beta Terpineol	2	71	93	136	
3.2.45	Menthol	1	71	95	81	
3.3.1	1,4-Dibromobenzene (**IS_A**)	1&2	236	238	234	
3.2.46	Methyl salicylate	2	120	152	92	
3.2.47	Methyl-2-octynoate (Folione®)	1	123	95	111	
3.2.55	alpha Terpineol	2	59	121	93	
3.2.55.a	gamma Terpineol	2	121	93	136	
3.2.19	Citronellol	1	69	81	95	
3.2.14	Carvone	2	82	54	108	
3.2.18B	Neral	1	69	119	84	
3.2.16	Cinnamaldehyde	1	131	103	132	
3.2.31	Geraniol	2	69	93	123	
3.2.42	Linalyl acetate	2	93	80	121	
3.2.18A	Geranial	1	84	83	152	
3.2.7	Anise alcohol	2	138	137	109	
3.2.35	Hydroxycitronellal	1	59	71	95	
3.2.6	trans Anethole	2	147	117	148	
3.2.17	Cinnamyl alcohol	1	92	115	78	
3.2.25	DMBC acetate (DiMethylBenzylCarbinyl)	2	132	117	91	
3.2.27	Eugenol	2	164	149	103	
3.2.57	Vanillin	2	151	152	81	
3.2.24	delta Damascone	1	69	192	123	
3.2.23	beta Damascone	1	177	192	123	

	OV1-type column					
	Identification	SIM Method	I1 (Da)	I2 (Da)	I3 (Da)	Internal Standard
3.2.32	Geranyl acetate	2	93	68	136	
3.2.22	alpha Damascone	1	69	192	123	
3.2.20	Coumarin	2	118	146	89	
3.2.44	Trimethyl-benzenepropanol (Majantol®)	2	106	91	105	
3.2.21	beta Damascenone	1	69	175	190	
3.2.15	beta Caryophyllene	2	93	91	133	
3.2.37	Isoeugenol	2	164	149	131	
3.2.26A	Ebanol 1	1	108	149	164	
3.2.26B	Ebanol 2	1	108	149	164	
3.2.38	alpha Isomethylionone	1	135	206	150	
3.2.28	Eugenyl acetate	1	164	149	131	
3.2.39	Butylphenyl methylpropional (Lilial®)	2	189	147	204	
3.2.50A	Propylidene phthalide(major)	2	159	174	104	
3.2.3	Amyl salicylate	1	120	138	208	
3.2.50B	Propylidene phthalide (minor)	2	159	174	104	
3.2.2	Acetyl isoeugenol / Isoeugenyl acetate	1	164	131	149	
3.2.4	alpha Amylcinnamaldehyde (Flosal®)	2	129	201	202	
3.2.43A	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (major)	2	136	192	177	
3.2.43B	Hydroxyisohexyl 4-cyclohexene carboxaldehyde (HICC) (Lyral®) (minor)	2	136	192	177	
3.2.36B	major beta Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	121	109	**IS_B**
3.2.36A	alpha Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	121	109	
3.2.29C	(*Z*,*Z*)-Farnesol	2	69	81	93	
3.2.5	alpha Amylcinnamyl alcohol	1	133	204	115	
3.2.52A	alpha Santalol	2	93	94	122	
3.2.36C	gamma Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	135	121	
3.2.29B	(*Z*,*E*)-Farnesol/(*E*,*Z*)-Farnesol	2	69	81	93	
3.2.52B	beta Santalol	2	93	94	122	
3.2.29A	(*E*,*E*)-Farnesol	2	69	81	93	
3.2.34	alpha Hexylcinnamaldehyde (Jasmonal®)	1	129	216	215	
3.2.10	Benzyl benzoate	1	105	212	194	
3.2.1	alpha Acetyl cedrene (Vertofix® main)	2	161	231	246	
3.2.12	Benzyl salicylate	1	91	228	65	

	OV1-type column					
	Identification	SIM Method	I1 (Da)	I2 (Da)	I3 (Da)	Internal Standard
3.2.30AB	Galaxolide 1+2	1	243	213	258	
3.2.33	Hexadecanolactone / Dihydroambrettolide	1	83	97	111	
3.3.2	4,4'-Dibromobiphenyl (**IS_B**)	1&2	312	314	310	
3.2.11	Benzyl cinnamate	2	131	192	193	
3.2.53	Sclareol	2	95	177	109	

Table 8 - SIM ions (m/z) of allergens listed in the elution order on OV17-type column, corresponding SIM methods (1 or 2) and internal standard (IS_A or IS_B) used for quantification

	OV17-type column					
	Identification	SIM Method	I1 (Da	I2 (Da)	I3 (Da)	Internal Standard
3.2.48	alpha Pinene	1	93	92	91	IS_A
3.2.49	beta Pinene	1	93	92	91	
3.2.54	alpha Terpinene	2	136	79	121	
3.2.40	Limonene	2	68	93	67	
3.2.8	Benzaldehyde	1	106	105	51	
3.2.56	Terpinolene	2	93	136	121	
3.2.41	Linalool	2	93	71	80	
3.2.9	Benzyl alcohol	1	79	108	107	
3.2.51	Salicylaldehyde	1	122	121	65	
3.2.55b	cis-beta Terpineol	2	71	93	136	
3.2.55c	trans-beta Terpineol	2	71	93	136	
3.2.45	Menthol	2	71	95	81	
3.2.13	Camphor	2	95	108	81	
3.2.55	alpha Terpineol	1	59	121	93	
3.2.55.a	gamma-Terpineol	1	121	93	136	
3.2.19	Citronellol	2	69	81	95	
3.2.42	Linalyl acetate	1	93	80	121	
3.2.47	Methyl-2-octynoate (Folione®)	1	123	95	66	
3.2.31	Geraniol	2	69	93	123	
3.2.46	Methyl salicylate	2	120	152	121	
3.2.18B	Neral	1	69	94	109	
3.3.1	1,4-Dibromobenzene (ISA)	1 & 2	236	238	234	
3.2.18A	Geranial	1	84	83	152	
3.2.14	Carvone	2	82	54	108	
3.2.35	Hydroxycitronellal	2	59	71	95	
3.2.6	trans Anethole	1	147	117	148	
3.2.25	DMBC acetate (DiMethylBenzylCarbinyl)	1	132	117	91	
3.2.15	beta Caryophyllene	2	93	91	133	
3.2.32	Geranyl acetate	1	93	68	136	
3.2.24	delta Damascone	2	69	192	123	
3.2.16	Cinnamaldehyde	1	131	103	132	
3.2.7	Anise alcohol	2	138	137	109	
3.2.26A	Ebanol 1	1	108	149	164	
3.2.22	alpha Damascone	2	69	192	123	
3.2.17	Cinnamyl alcohol	1	92	115	78	
3.2.21	beta Damascenone	2	69	175	190	

	OV17-type column					
	Identification	SIM Method	I1 (Da	I2 (Da)	I3 (Da)	Internal Standard
3.2.26B	Ebanol 2	1	108	149	164	
3.2.27	Eugenol	1	164	149	103	
3.2.23	beta Damascone	2	177	192	123	
3.2.44	Trimethyl-benzenepropanol (Majantol®)	1	106	91	105	
3.2.38	alpha Isomethylionone	2	135	206	150	
3.2.37	Isoeugenol	1	164	149	131	
3.2.57	Vanillin	2	151	152	81	
3.2.39	Butylphenyl methylpropional (Lilial®)	1	189	147	204	
3.2.3	Amyl salicylate	2	120	138	208	
3.2.20	Coumarin	1	118	146	89	
3.2.28	Eugenyl acetate	2	164	149	131	
3.2.36B	major beta Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	121	109	
3.2.36A	alpha Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	121	109	
3.2.29C	(*Z*,*Z*)- Farnesol	2	69	81	93	
3.2.52A	alpha Santalol	2	93	94	122	
3.2.29B	(*Z*,*E*)-Farnesol	2	69	81	93	
3.2.29D	(*E*,*Z*)-Farnesol	2	69	81	93	
3.2.36C	gamma Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	135	150	
3.2.50A	3-Propylidene phthalide(major)	2	159	174	104	
3.2.4	alpha Amylcinnamaldehyde (Flosal®)	1	129	201	202	
3.2.29A	(*E*,*E*)-Farnesol	2	69	81	93	
3.2.2	Acetyl isoeugenol / Isoeugenyl acetate	1	164	131	149	
3.2.43B	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (major)	2	136	192	177	
3.2.52B	beta Santalol	1	93	94	122	
3.2.43A	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (major)	2	136	192	177	
3.2.5	alpha Amylcinnamyl alcohol	2	133	204	115	
32.50B	3-Propylidene phthalide (minor)	1	159	174	104	
3.2.1	alpha Acetyl cedrene (Vertofix® main)	1	161	231	246	
3.2.34	alpha Hexylcinnamaldehyde (Jasmonal®)	2	129	216	215	
3.2.30A	Galaxolide 1	1	243	213	258	
3.2.30B	Galaxolide 2	1	243	213	258	
3.2.10	Benzyl benzoate	2	105	212	194	

	OV17-type column					
	Identification	SIM Method	I1 (Da	I2 (Da)	I3 (Da)	Internal Standard
3.2.33	Hexadecanolactone/Dihydroambrettolide	1	83	97	111	
3.2.12	Benzyl salicylate	2	91	228	65	
3.3.2	4,4'-Dibromobiphenyl (IS_B)	1 & 2	312	314	310	
3.2.53	Sclareol	1	95	177	109	
3.2.11	Benzyl cinnamate	2	131	192	193	

Table 9 - Alternative SIM ions (m/z) of allergens listed in the elution order on OV1-type column, corresponding SIM methods (1 or 2) and internal standard (IS_A or IS_B) used for quantification

NOTE – These should be validated by the Operator before use in the Procedure

All the target constituents where one or more of the SIM ions have been changed versus Table 7 are written in red.

	OV1-type column					
	Identification	SIM Method	I1 (uma)	I2 (uma)	I3 (uma)	Internal Standard
3.2.8	Benzaldehyde	1	106	105	51	
3.2.48	alpha Pinene	2	93	92	91	
3.2.49	beta Pinene	2	93	92	91	
3.2.9	Benzyl alcohol	1	**108**	**107**	**79**	
3.2.54	alpha Terpinene	2	136	79	121	
3.2.51	Salicylaldehyde	1	**122**	**121**	**104**	
3.2.40	Limonene	2	68	93	67	
3.2.56	Terpinolene	2	**136**	**121**	**91**	
3.2.41	Linalool	2	**71**	**121**	**55**	
3.2.13	Camphor	1	95	108	81	
3.2.55B	cis-beta Terpineol	2	71	93	136	
3.2.55C	trans-beta Terpineol	2	71	93	136	
3.2.45	Menthol	1	71	95	81	
3.3.1	1,4-Dibromobenzene (**IS_A**)	1 & 2	236	238	234	
3.2.46	Methyl salicylate	2	**152**	**120**	**92**	
3.2.47	Methyl-2-octynoate (Folione®)	1	**123**	**95**	**139**	
3.2.55A	alpha Terpineol	2	**121**	**59**	**93**	**IS_A**
3.2.19	Citronellol	1	69	81	95	
3.2.14	Carvone	2	**82**	**93**	**108**	
3.2.18B	Neral	1	**84**	**94**	**137**	
3.2.16	Cinnamaldehyde	1	**131**	**132**	**103**	
3.2.31	Geraniol	2	69	93	123	
3.2.42	Linalyl acetate	2	93	80	121	
3.2.18A	Geranial	1	**84**	**94**	**152**	
3.2.7	Anise alcohol	2	138	137	109	
3.2.35	Hydroxycitronellal	1	**71**	**96**	**139**	
3.2.6	trans Anethole	2	**148**	**147**	**117**	
3.2.17	Cinnamyl alcohol	1	**134**	**92**	**91**	
3.2.25	DMBC acetate (DiMethylBenzylCarbinyl)	2	132	117	91	
3.2.27	Eugenol	2	164	149	103	
3.2.57	Vanillin	2	**151**	**152**	**123**	
3.2.24	delta Damascone	1	69	192	123	

	OV1-type column					
	Identification	SIM Method	I1 (uma)	I2 (uma)	I3 (uma)	Internal Standard
3.2.23	beta Damascone	1	177	192	123	
3.2.32	Geranyl acetate	2	**136**	**93**	**121**	
3.2.22	alpha Damascone	1	69	192	123	
3.2.20	Coumarin	2	**118**	**146**	**90**	
3.2.44	Trimethyl-benzenepropanol (Majantol®)	2	106	91	105	
3.2.21	beta Damascenone	1	**190**	**175**	**105**	
3.2.15	beta Caryophyllene	2	133	91	93	
3.2.37	Isoeugenol	2	164	149	131	
3.2.26A	Ebanol 1	1	**164**	**108**	**121**	
3.2.26B	Ebanol 2	1	**164**	**108**	**121**	
3.2.38	alpha Isomethylionone	1	135	206	150	
3.2.28	Eugenyl acetate	1	**164**	**131**	**206**	
3.2.39	Butylphenyl methylpropional (Lilial®)	2	189	147	204	
3.2.50A	Propylidene phthalide (major)	2	159	174	104	
3.2.3	Amyl salicylate	1	**138**	**208**	**121**	
3.2.50B	Propylidene phthalide (minor)	2	159	174	104	
3.2.2	Acetyl isoeugenol / Isoeugenyl acetate	1	164	131	149	
3.2.4	alpha Amylcinnamaldehyde (flosal®)	2	129	201	202	
3.2.43A	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (major)	2	136	192	177	
3.2.43B	Hydroxyisohexyl 4-cyclohexene carboxaldehyde (HICC) (Lyral®) (minor)	2	136	192	177	
3.2.36B	major beta Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	**191**	**119**	**121**	**IS_B**
3.2.36A	alpha Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	191	121	109	
3.2.5	alpha Amylcinnamyl alcohol	1	**133**	**115**	**129**	
3.2.52A	alpha Santalol	2	**93**	**122**	**107**	
3.2.36C	gamma Tetramethylacetyloctahydronaphthalene (ISO E® Super)	1	**191**	**135**	**150**	
3.2.29B	(*Z,E*)-Farnesol	2	**69**	**93**	**136**	
3.2.52B	beta Santalol	2	**94**	**122**	**107**	
3.2.29A	(*E,E*)-Farnesol	2	69	81	93	
3.2.34	alpha Hexylcinnamaldehyde (Jasmonal®)	1	**129**	**216**	**215**	
3.2.10	Benzyl benzoate	1	105	212	194	
3.2.1	alpha Acetyl cedrene (Vertofix® main)	2	161	231	246	
3.2.12	Benzyl salicylate	1	91	228	65	

	OV1-type column					
	Identification	SIM Method	I1 (uma)	I2 (uma)	I3 (uma)	Internal Standard
3.2.30AB	Galaxolide 1+2	1	243	213	258	
3.2.33	Hexadecanolactone/Dihydroambrettolide	1	**83**	**97**	**236**	
3.3.2	4,4'-Dibromobiphenyl (**IS_B**)	1 & 2	312	314	310	
3.2.11	Benzyl cinnamate	2	131	192	193	
3.2.53	Sclareol	2	**177**	**191**	**290**	

APPENDIX D: EXAMPLE OF SIM WINDOWS

Table 10 - Example of windows for SIM1 method on OV1 column
SIM Ions from Table 7
(Refer to **Appendix B** for characteristics and oven temperature)

METHOD : OV1-SIM1				RECORDED IONS							
Start time	**dwell**	**Name**	**cycle / sec**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0	50	Benzaldehyde (3.2.8)	5.8	51	105	106					
4.2		*no recording*									
4.8	50	Benzyl alcohol (3.2.9)	3.1		79	107	108				
	50	Salicylaldehyde (3.2.51)		65				121	122		
5.8		*no recording*									
7.1	50	Camphor (3.2.13)	5.8	81	95	108					
8.0	50	Menthol (3.2.45)	3.1	71	81	95					
	50	Methyl-2-octynoate (Folione®) (3.2.47)				95	111	123			
	50	1,4-Dibromobenzene – IS_A (3.3.1)							236	234	238
9.5	50	Citronellol (3.2.19)	3.7	69	81	95					
	50	Neral (3.2.18B)		69			84	119			
10.2	50	Cinnamaldehyde (3.2.16)	3.1			103	131	132			
	50	Geranial (3.2.18A)		83	84				152		
11	50	Hydroxycitronellal (3.2.35)	3.1	59	71			95			
	50	Cinnamyl alcohol (3.2.16)				78	92		115		
13.5	50	delta Damascone (3.2.24)	3.1	69	123				192		
	50	beta Damascenone (3.2.21)		69		175		190			
	50	alpha Damascone (3.2.22)		69	123				192		
	50	beta Damascone (3.2.24)			123		177		192		
15.5	50	Ebanols (3.2.26)	5.8	108	149	164					
16.9	50	alpha Isomethylionone (3.2.38)	3.1		135		150		206		
	50	Eugenyl acetate (3.2.28)		131		149		164			
18.5	50	Amyl salicylate (3.2.3)	5.8	120	138	208					
20	50	Acetyl isoeugenol (3.2.2)	5.8	131	149	164					
21	44	beta Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36B)	3	109		121			191		
	44	alpha Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36A)		109		121			191		
	44	gamma Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36C)				121		135	191		
	44	alpha Amylcinnamyl alcohol (3.2.5)			115		133			204	
22.6	50	alpha Hexylcinnamaldehyde (3.2.34)	3.1		129			215	216		
	50	Benzyl benzoate (3.2.10)		105		194	212				
23.6	50	Benzyl salicylate (3.2.12)	3.1	65	91		228				
	50	Galaxolide (3.2.30)				213		243	258		

METHOD : OV1-SIM1				RECORDED IONS					
25	50	Hexadecanolactone (3.2.33)	3.1	83	97	111			
	50	4,4'-Dibromobiphenyl (IS_B) (3.3.2)					312	310	314
27		*no recording*							

Table 11 - Example of windows for SIM2 method on OV1 column
SIM Ions from Table 7
(Refer to **Appendix B** for characteristics and oven temperature)

METHOD : OV1-SIM2				RECORDED IONS							
Start time	**dwell**	**Name**	**cycle / sec**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0	50	alpha Pinene (3.2.48)	5.8	91	93	92					
	50	beta Pinene (3.2.49)		91	93	92					
5	50	alpha Terpinene (3.2.54)	3.1			79		121	136		
	50	Limonene (3.2.40)		67	68		93				
6.2	50	Terpinolene (3.2.56)	3.7			93	121	136			
	50	Linalool (3.2.41)		71	80	93					
7.3	50	beta Terpineol (3.2.55b & c)	5.8	71	93	136					
8.3	44	1,4-Dibromobenzene (IS_A) (3.3.1)							236	234	238
	44	Methyl salicylate (3.2.46)	3		92		120		152		
	44	alpha Terpineol (3.2.55)		59		93		121			
9.6	50	Carvone (3.2.14)	5.8	54	82	108					
10.3	38	Geraniol (3.2.31)		69		93			123		
	38	Linalyl acetate (3.2.42)	3		80	93		121			
	38	Anise alcohol (3.2.7)					109			137	138
11.2	50	Anethole (3.2.6)	3.7		117		147	148			
	50	DMBC acetate (3.2.25)		91	117	132					
13	50	Eugenol (3.2.27)	3.1		103	149			164		
	50	Vanillin (3.2.57)		81			151	152			
14.2	50	Geranyl acetate (3.2.32)	5.8	68	93	136					
14.7	50	Coumarin (3.2.20)	3.1	89				118	146		
	50	Trimethyl-benzenepropanol (Majantol®) (3.2.44)			91	105	106				
15.6	50	beta Caryophyllene (3.2.15)	3.1	91	93		133				
	50	Isoeugenol (3.2.37)				131		149	164		
16.3		*no recording*									
17.6	50	Butylphenyl methylpropional (Lilial®) (3.2.39)	3.1		147			189	204		
	50	Propylidene phthalide, 3- (3.2.50)		104		159	174				
20.6	50	alpha Amylcinnamaldehyde (3.2.4)	3.1	129				201	202		
	50	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43)			136	177	192				
21.7	50	Santalol (3.2.52)	3.7			93	94	122			
	50	Farnesol (3.2.29)		69	81	93					
23	50	alpha Acetyl cedrene (3.2.1)	5.8	161	231	246					
23.8		*no recording*									
25	50	4,4'-Dibromobiphenyl (IS_B) (3.3.2)	4.5				312	310	314		
	50	Benzyl cinnamate (3.2.11)		131	192	193					
27.6	50	Sclareol (3.2.53)	5.8	95	109	177					

Table 12 - Example of windows for SIM1 method on OV17 column
SIM Ions from Table 8
(Refer to **Appendix B** for characteristics and oven temperature)

METHOD : OV17-SIM1				RECORDED IONS							
Start time	**dwell**	**Name**	**cycle / sec**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0	50	alpha Pinene (3.2.48)	4.5	91	92	93					
	50	beta Pinene (3.2.49)		91	92	93					
3.3		*no recording*	8.3	900							
4	50	Benzaldehyde (3.2.8)	5.8	51	105	106					
4.9	50	Benzyl alcohol (3.2.9)	3.1		79	107	108				
	50	Salicylaldehyde (3.2.51)		65				121	122		
6.0	44	alpha Terpineol (3.2.55A)		59			93		121		
	44	Linalyl acetate (3.2.42)	3			80	93		121		
	44	Methyl-2-octynoate (Folione®) (3.2.47)			66			95		123	
7.1	44	Neral (3.2.18B)		69			94	109			
	44	1,4-Dibromobenzene (IS_A) (3.3.1)	3						234	236	238
	44	Geranial (3.2.18A)			83	84			152		
8.3	50	Anethole (3.2.6)	3.7		117		147	148			
	50	DMBC acetate (3.2.25)		91	117	132					
9.2	50	Geranyl acetate (3.2.32)	3.1	68	93				136		
	50	Cinnamaldehyde (3.2.16)				103	131	132			
10.0	44	Ebanols (3.2.26)					108		149	164	
	44	Cinnamyl alcohol (3.2.16)	3	78	92			115			
	44	Eugenol (3.2.27)				103			149	164	
11.2	50	Trimethyl-benzenepropanol (Majantol®) (3.2.44)	3.1	91	105	106					
	50	Isoeugenol (3.2.37)					131	149	164		
14.3	50	Butylphenyl methylpropional (Lilial®) (3.2.39)	3.1				147	189	204		
	50	Coumarin (3.2.20)		89	118	146					
17	50	beta Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36B)	5.8	109	121	191					
	50	alpha Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36A)		109	121	191					
18.3	50	gamma Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36C)	3.1		135	150	191				
	50	alpha Amylcinnamaldehyde (3.2.4)		129				201	202		
19.4	50	Acetyl isoeugenol (3.2.2)	3.1				131	149	164		
	50	Santalol (3.2.52)		93	94	122					
20.4	50	Propylidene phthalide, 3- minor (3.2.50B)	3.1	104	159		174				
	50	alpha Acetyl cedrene (3.2.1)				161		231	246		
23	50	Galaxolide (3.2.30)	3.1				213	243	258		
	50	Hexadecanolactone (3.2.33)		83	97	111					

METHOD : OV17-SIM1				RECORDED IONS					
27	50	4,4'-Dibromobiphenyl (IS_B) (3.3.2)	4.5				312	310	314
	50	Sclareol (3.2.53)		95	109	177			

Table 13 - Example of windows for SIM2 method on OV17 column
SIM Ions from Table 8
(Refer to **Appendix B** for characteristics and oven temperature)

METHOD : OV17-SIM2				RECORDED IONS							
Start time	**dwell**	**Name**	**cycle / sec**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
0		*no recording*									
3.2	50	alpha Terpinene (3.2.54)	3.1			79		121	136		
	50	Limonene (3.2.40)		67	68		93				
4	50	Terpinolene (3.2.56)	3.1			93	121	136	136		
	50	Linalool (3.2.41)		71	80	93					
5.1	44	beta Terpineol (3.2.55b & c)	3		71		93			136	
	44	Menthol (3.2.45)			71	81		95			
	44	Camphor (3.2.13)				81		95	108		
	44	Citronellol (3.2.19)		69		81	95				
6.8	44	Geraniol (3.2.31)	3	69	93			123			
	44	Methyl salicylate (3.2.46)				120	121		152		
	44	1,4-Dibromobenzene (IS_A) (3.3.1)							234	236	238
7.6	50	Carvone (3.2.14)	3.1	54			82		108		
	50	Hydroxycitronellal (3.2.35)			59	71		95			
9	50	beta Caryophyllene (3.2.15)	3.1		91	93		133			
	50	delta Damascone (3.2.24)		69			123		192		
9.9	38	Anise alcohol (3.2.7)	3		109	137		138			
	38	alpha Damascone (3.2.22) beta Damascone (3.2.23)		69			123				192
	38	beta Damascenone (3.2.21)		69					175	190	
11.3	50	alpha Isomethylionone (3.2.38)	3.1		135	150			206		
	50	Vanillin (3.2.57)		81			151	152			
15	50	Amyl salicylate (3.2.3)	3.1	120		138			208		
	50	Eugenyl acetate (3.2.28)			131		149	164			
17.3	38	Santalol (3.2.52)	3			93	94		122		
	38	Farnesol (3.2.29)		69	81	93					
	38	Propylidene phthalide, 3- major (3.2.50A)						104		159	174
19.5	50	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43)	3.1			136	177	192			
	50	alpha Amylcinnamyl alcohol (3.2.5)		115	133				204		
20.5	50	alpha Hexylcinnamaldehyde (3.2.34)	5.8	129	215	216					
23.5	50	Benzyl benzoate (3.2.10)	3.1			105	194	212			
	50	Benzyl salicylate (3.2.12)		65	91				228		
27	50	4,4'-Dibromobiphenyl (IS_B) (3.3.2)	3.1				312	310	314		
	50	Benzyl cinnamate (3.2.11)		131	192	193					

APPENDIX E: EXAMPLES OF CHROMATOGRAMS

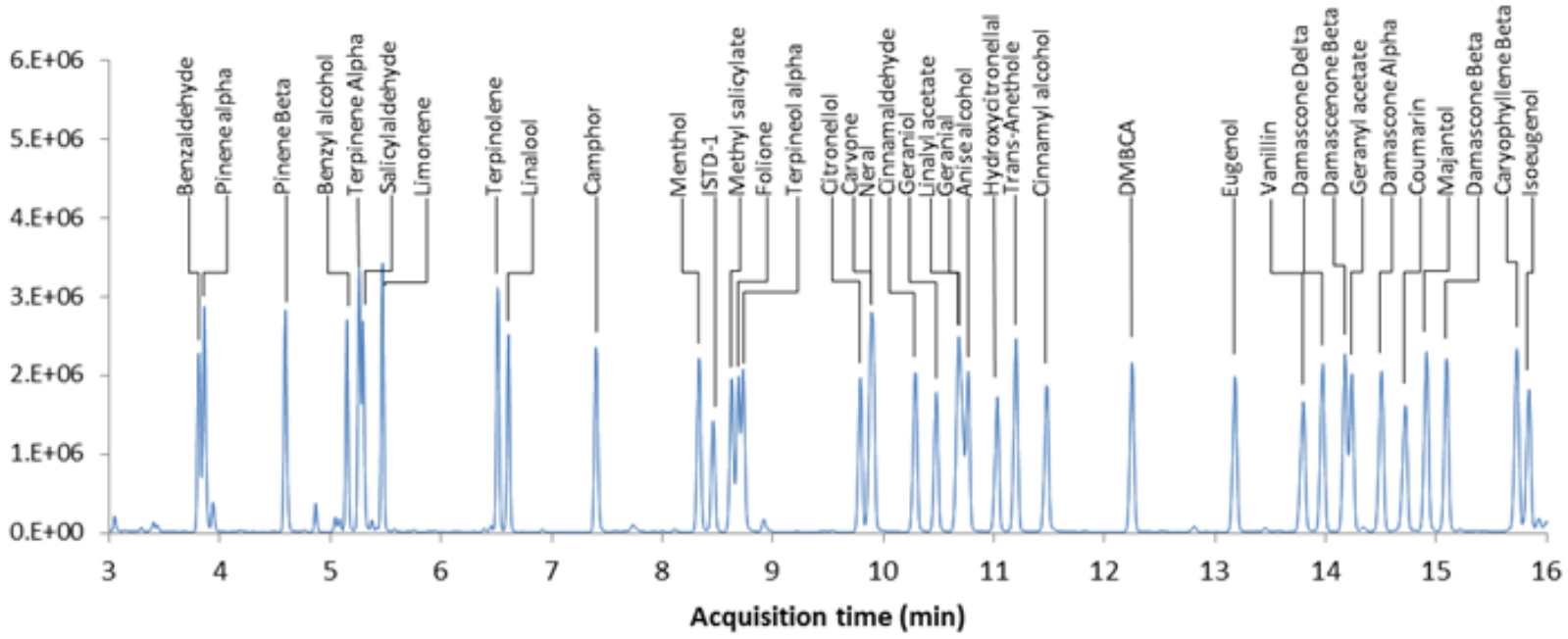


Figure 3 - Example of chromatogram for method on apolar column
(Refer to **Appendix B** for characteristics and oven temperature)

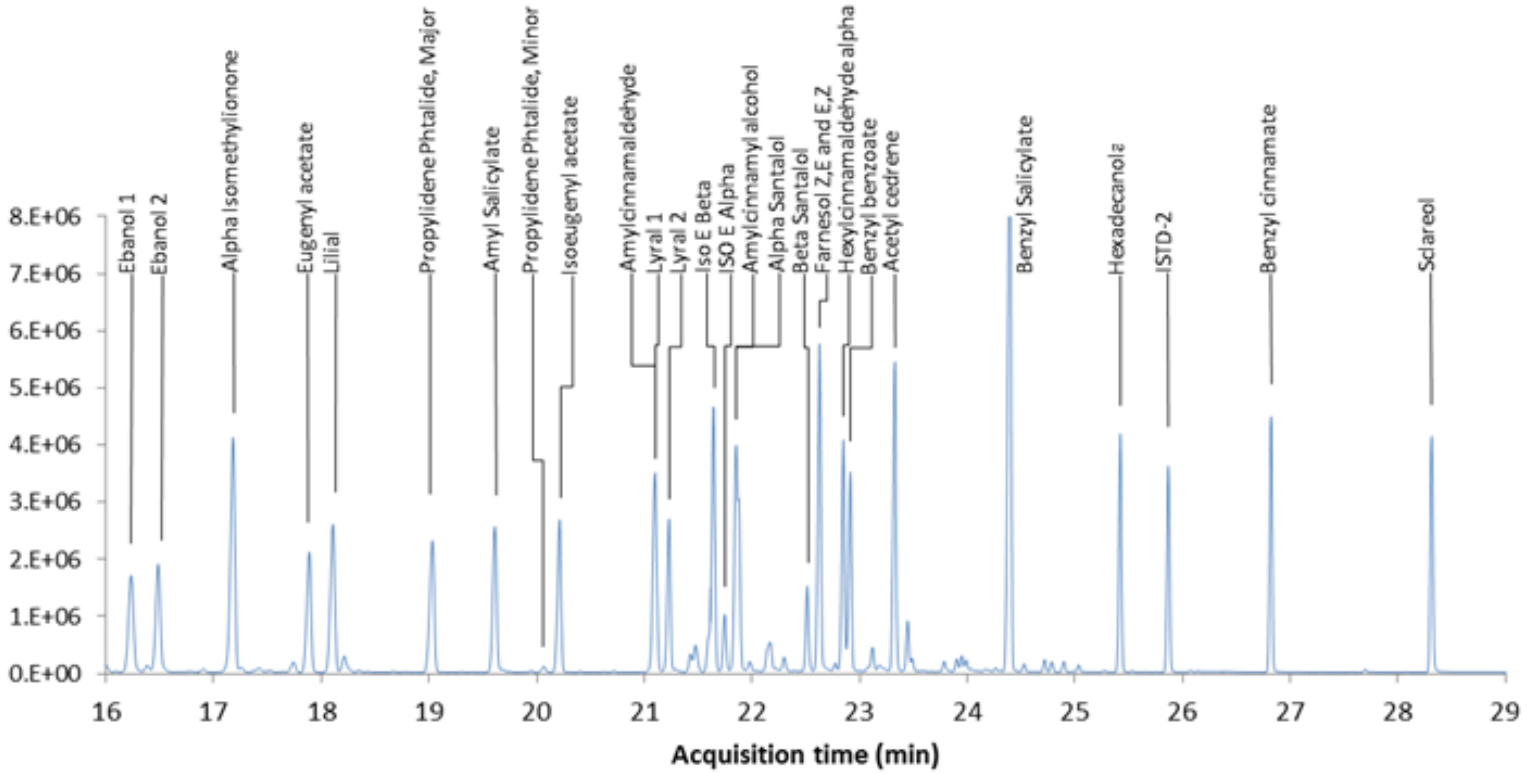


Figure 4 - Example of chromatogram for method on apolar column
(Refer to **Appendix B** for characteristics and oven temperature)

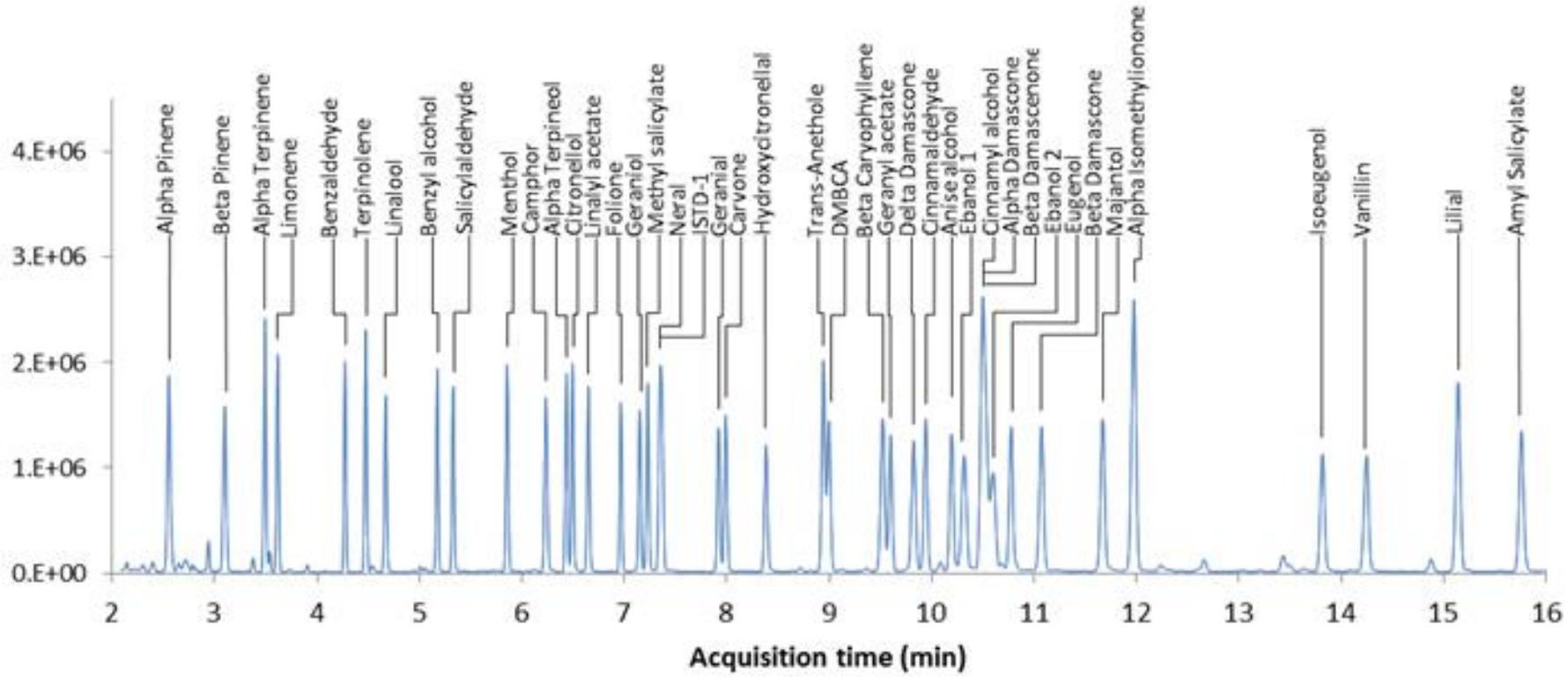


Figure 5 - Example of chromatogram for method on polar column
(Refer to **Appendix B** for characteristics and oven temperature)

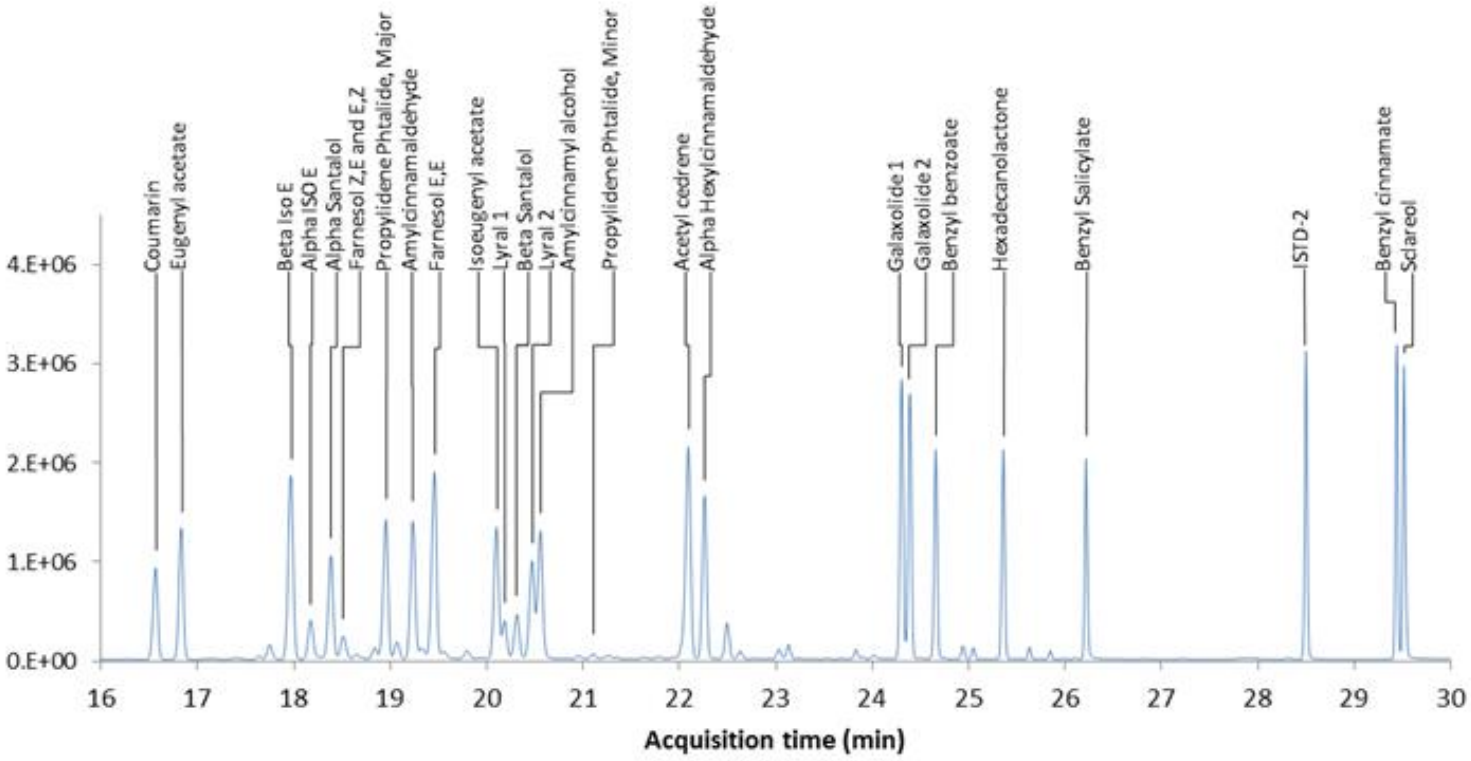


Figure 6 - Example of chromatogram for method on polar column
(Refer to **Appendix B** for characteristics and oven temperature)**!**

APPENDIX F: DECISIONAL TREE FOR QUANTIFICATION OF ALLERGENS

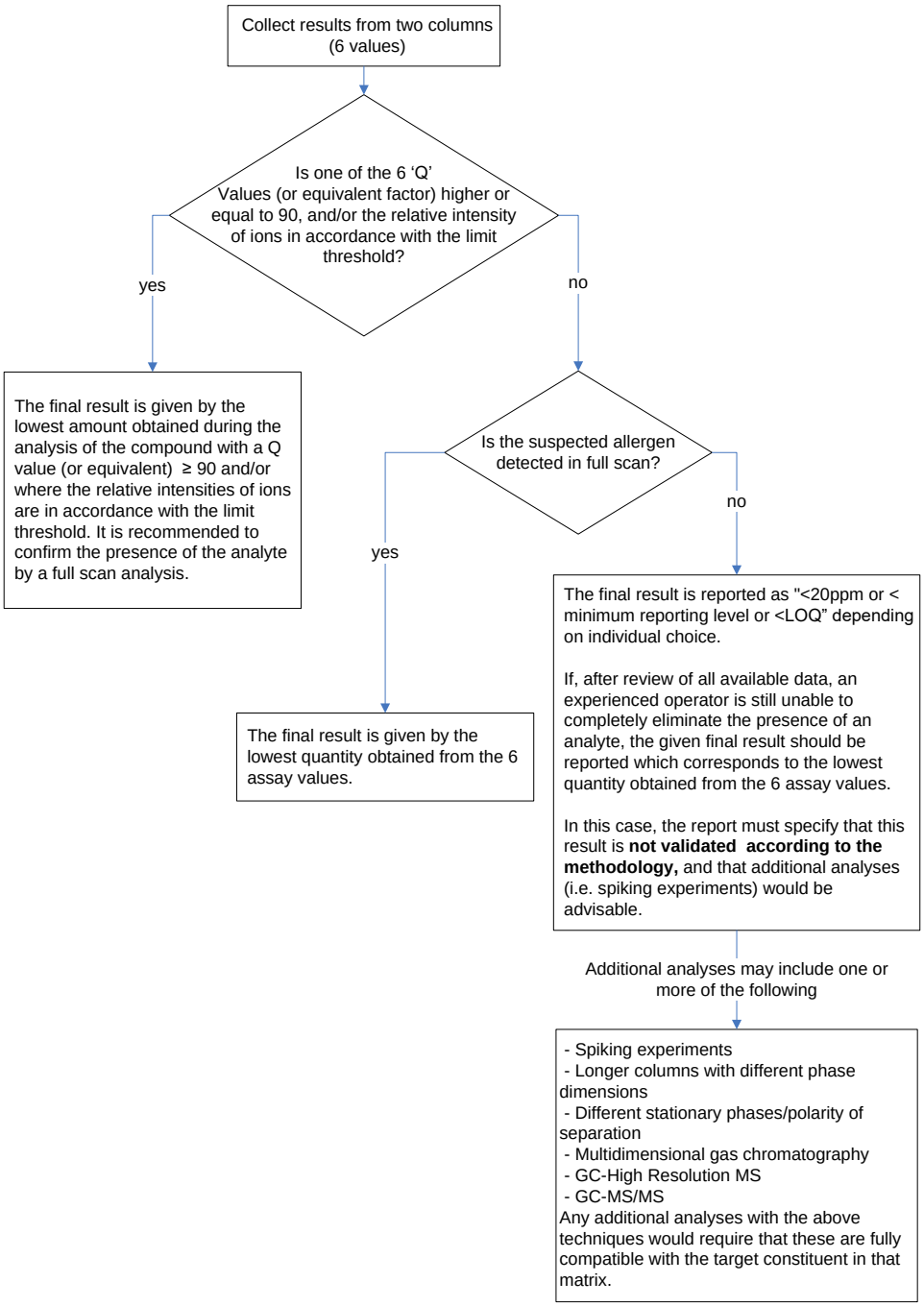


Figure 7 - Decisional tree for quantification of allergens.

APPENDIX G: PREPARATION OF STOCK SOLUTIONS FROM REFERENCE SAMPLES

G.1. General information

G.2. Reference sample purity

The purity of each standard and internal standard and the respective percentages of geometric isomers must be determined by GC-FID. The procedure to follow for the purity study is the subject of a detailed protocol presented in **Appendix A**. If the operator is using certified standards or stock solutions then the purity determination can be omitted.

Substances recognised to exist in several isomeric forms are:

- alpha Acetyl cedrene /Vertofix® main (3.2.1),
- alpha Amyl cinnamaldehyde/Flosal® (3.2.4),
- alpha Amylcinnamyl alcohol (3.2.5),
- Anethole (3.2.6), Benzyl cinnamate (3.2.11),
- Cinnamaldehyde (3.2.16),
- Cinnamyl alcohol (3.2.17), Citral (3.2.18),
- alpha Damascone (3.2.22),
- delta Damascone (3.2.24),
- Ebanol (3.2.26),
- Farnesol (3.2.29),
- Galaxolide/Hexamethylindanopyran(3.2.30),
- alpha Hexylcinnamaldehyde / Jasmonal® (3.2.34),
- Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36),
- Isoeugenol (3.2.37),
- alpha Isomethylionone (3.2.38),
- Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43),
- 3-Propylidene phthalide (3.2.50),
- Santalol (3.2.52),
- Terpineol (3.2.55).

The weighing of the reference standards is performed taking into account standard purity.

For most suspected allergens mixtures, quantification is based on the amount of the main isomer in the reference sample and therefore purity of the main isomer in the mixture is used to determine the amount of the reference sample to be weighed.

For binary mixtures (Citral (3.2.18), Ebanol (3.2.26), Galaxolide / Hexamethylindanopyran (3.2.30), Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43), 3-Propylidene phthalide (3.2.50) and Santalol (3.2.52)), it is recommended to double the concentrations described below (5.2.2) to be in accordance with the peak area of a single peak analyte. alpha Acetyl cedrene (3.2.1), Farnesol (3.2.29), Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36) and alpha Iso methyl ionone (3.2.38) are also to be treated in this way.

G.3. Stock solutions stability

To limit chemical reactions between analytes and degradation, two separated stock solutions are prepared in the dilution solvent:

- One stock solution contains alcohols and terpenes without carbonyl function,
- The other solution contains the remaining suspected allergens, in particular aldehydes and ketones.

The stability of substances in the stock solutions, prepared according to the protocol described below, was investigated in glass bottle in darkness and in a freezer at a temperature lower than -18°C. In these conditions, a 1 month stability period was established for the two stock solutions.

If the storage conditions differ from those described above, investigations must be performed to demonstrate the substances' stability in the new defined conditions.

G.3.1. Separated stock solutions of allergens (10g/kg)

G.3.1.1. Weighing

All weighing of reference samples and internal standards are carried out on a calibrated analytical balance with a readability of 0.0001g. Common laboratory glassware and equipment is used to prepare standard solutions and dilutions.

Prior to weighing, for liquid reference samples, homogenization is performed by vortex shaking. For those materials whose melting points are lower than 35°C:

- Anethole (3.2.6)
- Anise alcohol (3.2.7)
- Hexadecanolactone / Dihydroambrettolide (3.2.33)
- Menthol (3.2.45) – Note: this can be added as a solid depending on the purity of the material being used
- Amyl salicylate (3.2.3)
- Cinnamyl alcohol (3.2.17)
- Benzyl cinnamate (3.2.11)

These should be gently heated in a water bath and then treated as liquids or, alternatively, these can be directly added as solids if they are in a suitable form.

For guidance, it will be noted that in the tables below, the amount of an allergen to be weighed is variable. This is to compensate for the known purity or isomer composition of the individual material. From time to time, allergens of changed purity may be used. If this is the case with locally prepared calibration mixtures, then it is the responsibility of the analyst to ensure that appropriate amounts of each analyte are weighed with respect to the determined purity.

The mass of each allergen weighed must be recorded with the precision that the balance will allow but it is recognised that the mass of the final solution, where this is 1kg or more, may be beyond the range of a normal analytical balance. In this case it may be acceptable to use a balance of lower precision but this must be recorded.

G.3.1.2. Preparation

Two solutions are to be prepared, A1 and A2 using an appropriate solvent as described in 3.1 of the required purity. It is recommended that each allergen is weighed in a clean, dry weighing boat of the appropriate size and transferred to a weighed volumetric flask by rinsing with a small volume of solvent. After transfer, the residual solvent should be allowed to evaporate from the weighing boat which is then check weighed to ensure the transfer of the allergen is complete. Finally, the solution is made up to 1kg with solvent. Please note that the final volume of each solution is likely to be about 1143ml (0.875g/ml).

Prepare individual solutions of A1 and A2 by weighing the amounts of each substance as given in the following **Tables 14 and 15** into a suitable container and adjusting the final total solution weight with solvent to 1kg.

Note 21: these solutions can also be obtained by weighing accurately the mg equivalents of each compound in a flask and by adjusting the total mass of the solution to 10g with solvent. (For example, geranyl acetate, 100mg, diluted to 10g with solvent). Alternative approaches can be used concerning the weights used providing the final concentrations match those of the calibration table given in the Procedure.

Table 14 - Required mass for the preparation of Solution A1

Separated Stock Materials for Solution A1			**Required mass (g)**
3.2.5	alpha Amylcinnamyl alcohol	RN CAS [184900-07-0]	10
3.2.6	Anethole	RN CAS [4180-23-8]	10
3.2.7	Anise alcohol	RN CAS [105-13-5]	10
3.2.9	Benzyl alcohol	RN CAS [100-51-6]	10
3.2.15	beta Caryophyllene	RN CAS [87-44-5]	10
3.2.17	Cinnamyl alcohol	RN CAS [104-54-1]	10
3.2.19	Citronellol	RN CAS [106-22-9], [1117-61-9], [7540-51-4]	10
3.2.26	Ebanol	RN CAS [67801-20-1]	20
3.2.27	Eugenol	RN CAS [97-53-0]	10
3.2.29	Farnesol	RN CAS [4602-84-0]	20
3.2.31	Geraniol	RN CAS [106-24-1]	10
3.2.37	Isoeugenol	RN CAS [97-54-1]	10
3.2.40	Limonene	RN CAS [5989-27-5]	10
3.2.41	Linalool	RN CAS [78-70-6]	10
3.2.44	Trimethyl-benzenepropanol (Majantol®)	RN CAS [103694-68-4]	10
3.2.45	Menthol	RN CAS [1490-04-6], [89-78-1], [2216-51-5]	10
3.2.48	alpha Pinene	RN CAS [80-56-8]	10
3.2.49	beta Pinene	RN CAS [127-91-3]	10
3.2.52	Santalol	RN CAS [11031-45-1]	20
3.2.53	Sclareol	RN CAS [515-03-7]	10
3.2.54	alpha Terpinene	RN CAS [99-86-5]	10
3.2.55	Terpineol	RN CAS [98-55-5]	10

Table 15 - Required mass for the preparation of Solution A2

Separated Stock Materials for Solution A2			Required mass (g)
3.2.1	alpha Acetyl cedrene (Vertofix® main)	RN CAS [32388-55-9]	20
3.2.2.	Acetyl isoeugenol / Isoeugenyl acetate	RN CAS [93-29-8]	10
3.2.3	Amyl salicylate (n-Amyl salicylate)	RN CAS [2050-08-0]	10
3.2.4	alpha Amyl cinnamaldehyde (Flosal®)	RN CAS [78605-96-6]	10
3.2.8	Benzaldehyde	RN CAS [100-52-7]	10
3.2.10	Benzyl benzoate	RN CAS [120-51-4]	10
3.2.11	Benzyl cinnamate	RN CAS [103-41-3];	10
3.2.12	Benzyl salicylate	RN CAS [118-58-1]	10
3.2.13	Camphor	RN CAS [76-22-2], 464-49-3]	10
3.2.14	Carvone	RN CAS [99-49-0], [6485-40-1], [2244-16-8]	10
3.2.16	Cinnamaldehyde	RN CAS [104-55-2]	10
3.2.18	Citral	RN CAS [5392-40-5], isomers: RN CAS [106-26-3], [141-27-5]	20
3.2.20	Coumarin	RN CAS [91-64-5]	10
3.2.21	beta Damascenone (Rose Ketone-4)	RN CAS [23696-85-7]	10
3.2.22	alpha Damascone	RN CAS [43052-87-5]	10
3.2.23	beta (E)Damascone	RN CAS [31191-93-2]	10
3.2.24	delta Damascone (Rose Ketone-3)	RN CAS [57378-68-4]	10
3.2.25	Dimethylbenzylcarbinyl acetate (DMBCA)	RN CAS [151-05-3]	10
3.2.28	Eugenyl acetate	RN CAS [93-28-7]	10
3.2.30	Galaxolide (Hexamethylindanopyran)	RN CAS [1222-05-5]	20
3.2.32	Geranyl acetate	RN CAS [105-87-3]	10
3.2.33	Hexadecanolactone / Dihydroambrettolide	RN CAS [109-29-5]	10
3.2.34	alpha Hexylcinnamaldehyde (Jasmonal®)	RN CAS [101-86-0]	10
3.2.35	Hydroxycitronellal	RN CAS [107-75-5]	10
3.2.36	Tetramethylacetyloctahydronaphthalene (ISO E® super)	RN CAS [68155-66], RN CAS [54464-57-2], RN CAS [68155-67-9].	20
3.2.38	alpha Isomethylionone	RN CAS [127-51-5]	20
3.2.39	Butylphenyl methylpropional (Lilial®)	RN CAS [80-54-6]	10
3.2.42	Linalyl acetate	RN CAS [115-95-7]	10
3.2.43	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®)	RN CAS [31906-04-4]	13

Separated Stock Materials for Solution A2			**Required mass (g)**
3.2.46	Methyl salicylate	RN CAS [119-36-8]	10
3.2.47	Methyl-2-octynoate (Folione®)	RN CAS [111-12-6]	10
3.2.50	3-Propylidene phthalide	RN CAS [17369-59-4] ((E)-isomer: RN CAS [56014-72-3] and (Z)-isomer: [94704-89-9])	12
3.2.51	Salicylaldehyde	RN CAS [90-02-8]	10
3.2.57	Terpinolene	RN CAS [586-62-9]	10
3.2.58	Vanillin	RN CAS [121-33-5]	10

APPENDIX H: Calibration Methods and Approach

H.1. Plotting of calibration curve

Inject each calibration solution (5.2.5) on the two different columns according to the two SIM or SIM-SCAN methods. For each allergen compound, each ion in **Tables 7-9 (Appendix C)** can be used as the quantifier ion, whilst the two remaining ions being used as qualifier ions. Consequently, up to 3 calibration curves per column type can be constructed from the injection of the standard solutions, giving six different calibration curves for each allergen. If the calibration is compromised, for example by the coelution of a common ion, then one of the qualifier ions may be used to achieve the result.

In each case, build the calibration curves by plotting the area ratio between the analyte and the internal standard as a function of their concentration ratio:

$$\frac{area_i}{area_{IS}} = f\left(\frac{C_i}{C_{IS}}\right)$$

Where:
$area_i$ is the SIM area of compound I,
$area_{IS}$ is the area of the internal standard.
C_i and C_{IS} are, respectively, the allergen and internal standard concentration in each peak area measured by GC-MS for the quantifier ion.

Use a Quadratic Fit: Force through zero model (forced through the origin) to check the linearity domain of each calibration curve. In addition, a weighting factor may be applied to the calibration curve of 1/Concentration to further improve the performance of the method. This should be assessed by the Operator for effectiveness in use.

Examine the determination coefficient R^2 and the residuals (bias between theoretical and experimental concentration values) accordingly. An R^2 lower than 0.995 may be correlated to factors such as contamination of the injector liner, the MS source, the column or problems encountered during the calibration solution preparation. For individual residuals, deviation by more than ± 20% from the theoretical value (± 30% at the lowest calibration level) is considered as being unacceptable.

H.1.1. Peak Skewness and Impact on Calibration

Linearity may be compromised by peak skewness. To determine this effect, measure the peak width at 5% of the peak height above the baseline, drop a perpendicular from the peak apex to this line and determine the ratio of the two intercepts. Acceptance Limits of 0.8 to 1.2 have been proposed.

H.1.2. Selection of the internal standard

To identify the internal standard used for each analyte calibration, 1,4-Dibromobenzene (IS_A) or 4,4'-Dibromobiphenyl (IS_B), divide the retention time axis in two parts by calculating the median retention time (RT_{median}) as follows :

$$RT_{median} = RT_{ISA} + \left[\frac{(RT_{ISB} - RT_{ISA})}{2}\right]$$

Where:
RT_{ISA} and RT_{ISB} are respectively retention times of 1,4-Dibromobenzene and 4,4'-Dibromobiphenyl.

Use 1,4-dibromobenzene as internal standard for allergens that elute before RT_{median} and 4,4'-Dibromobiphenyl as internal standard for allergens that elute after RT_{median} (See **Tables 7-9 in Appendix C**). Use the ion at m/z 236 as the quantifier for 1,4-Dibromobenzene (IS_A) and the ion at m/z 312 as quantifier for 4,4'-Dibromobiphenyl (IS_B).

H.1.3. Selection of the internal standard

Where a suspected allergen is a mixture of isomers, construct the calibration curve for the major isomer except for

- Citral (3.2.18), Ebanol (3.2.26), Galaxolide (3.2.30), Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43), 3-Propylidene phthalide (3.2.50), and Santalol (3.2.52), for which calibration curves should be constructed for the two isomers using the relative isomer ratios from either the commercial Certified Calibration Standard mixture or through an FID analysis to establish the relative % purity of each isomer.

- Farnesol (3.2.29) for which calibration curves should be constructed only for the (*E,E*) isomer; the remaining isomers are quantified by applying the calibration curve for the (*E,E*) isomer to the other measured isomer peaks. Note that the abundance of the other Farnesol isomers is dependent on the concentration and source in the sample – in some situations only the (*E,E*)- isomer may be seen.

- Tetramethylacetyloctahydronaphthalene (ISO E® Super) (3.2.36) for which calibration curves should be constructed for the beta isomer.

- Terpineol(s) – alpha Terpineol is typically the dominant isomer but the legislation requires a total Terpineol content to be determined. This is achieved through the summation of the total concentration of the other Terpineol isomers if present – cis-beta Terpineol; trans-beta Terpineol and gamma Terpineol.

APPENDIX I: Quantification of allergens

I.1. General Information

For all analytes on the two GC columns and for each of the three ions, the concentration of the allergen is determined from the results of the analysis performed in SIM, SIM-SCAN or SCAN modes, with internal standardization and using the calibration curves obtained in quadratic fit force through zero across the two column polarities.

Note: In addition, a weighting of 1/concentration of the calibration point may also be applied though this is optional.

The analyte concentrations are reported in mg/kg, this calculation taking into account the dilution factor applied in the sample preparation.

When the analyte is an isomer mixture with more than 2 constituents, determine the final amount by quantifying the major isomer and then evaluating the sum of isomers using a safety factor. Exceptions are:

- Citral (3.2.18)
- Ebanol (3.2.26)
- Galaxolide (3.2.30)
- Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®) (3.2.43)
- 3-Propylidene phthalide (3.2.50)
- Santalol (3.2.52)

These are to be quantified from the sum of their two isomers measured separately.

Calculate the concentration C_{Ix} (mg/kg) of one analyte in sample as follows:

$$C_{Ix} = C_0 \times \frac{m_{DIL}}{m}$$

Where:
C_0 is the allergen concentration in the injected vial (mg/kg), calculated from analysis of the ion at m/z = I_x (x = 1, 2 or 3) on both columns,
m_{DIL} is the mass of the diluted sample solution (mg) (see Chapter 8),
m the sample mass (mg).

I.2. Determination of C_0

C_0 is usually directly provided by the instrument software but can also be calculated from raw data depending on the regression chosen for the calibration.

In the case of quadratic regression force trough zero, the equation of the calibration will be:

$$\frac{area_i}{area_{IS}} = a \times \left(\frac{C_i}{C_{IS}}\right)^2 + b \times \frac{C_i}{C_{IS}}$$

Where:
i is the allergen,
IS is the internal standard,
C is the concentration in mg/kg,
area is the area of the quantifying ion.

The calibration curve can be obtained from the following quadratic formula:

$$a \times \left(\frac{C_i}{C_{IS}}\right)^2 + b \times \frac{C_i}{C_{IS}} - \frac{area_i}{area_{IS}} = 0$$

In the injected vial, C_i is equivalent to C_0.

To solve this equation, the discriminated value Δ must be calculated as follows:

$$\Delta = b^2 + 4a\left(\frac{area_i}{area_{IS}}\right)$$

According to the sign of Δ, different roots can solve the equation and can be calculated according to **Table 16** -

Table 16 - Resolution of Δ for the determination of C_0

Δ	**Roots**
> 0	$\frac{-b+\sqrt{\Delta}}{2a}$ and $\frac{-b-\sqrt{\Delta}}{2a}$
0	$\frac{-b}{2a}$
< 0	Not applicable (2 complex roots)

This will be used to calculate C_0 following the equation:

$$C_0 = R * C_{IS}$$

Where:
C_0 is the concentration of the analyte in the injected vial (mg/kg),
C_{IS} is the concentration of the internal standard in the injected vial (mg/kg),
R is the unique root of the quadratic formula for Δ=0 and the positive root for Δ > 0.

I.3. Assessment of the analytical measurement

The analytical measurement is accepted if the determined analyte concentration is greater than 20mg/kg in sample.

Due to the potential complexity of test samples for which the present method is addressed, the relevance of calculated concentration should be carefully studied. Different strategies can be applied depending on the operator's knowledge and /or the functionalities of the software used.

The choice of the final concentration should be performed in accordance with the requirements of the decision tree in **Appendix F**.

The presence of the analyte must be verified by one of the two values of Q (Q ≥ 90, see below), or by one of the six couples of ion ratios within the tolerances allowed (allowable Ion Tolerances – see Table 17).

I.4. Examination of Q values

Calculate the Q value from the peak area of the 3 different ions I1, I2 and I3, as follows:

$$Q = 100 - \frac{\sum_{i=1}^{i=n}(100\times|r_i - r_i'^1|)(ln[100r_i+1])}{21.3\times\sum_{i=1}^{i=n} r_i}^2$$

Where:
n is the number of qualifier ions for each analyte,
r_i is the reference area ratio and
r_i' is the experimental area ratio.

The identity of the suspected allergen is confirmed if one (at least) of the 3 Q values per column is higher or equal to 90. If the Q value is zero, the corresponding concentration value should not be kept.

It is recommended to determine the reference area ratio from the injection of a standard solution at a concentration in the mid-range of the calibration curve.

I.4.1. Maximum tolerance for relative intensity of ions

To identify an allergen, compare the relative abundances of ions in the sample to the ones calculated from a standard solution. The allowable tolerances for the relative ion intensities (expressed in percentage of intensity of the most abundant ion) are given in Table 17.

Table 17 - Allowable Ion Tolerances.

Relative intensity	Threshold values
> 50%	± 10%
> 20% to 50%	± 15%
> 10% to 20%	± 20%
≤ 10%	± 50%

APPENDIX J: ALLERGENS SELECTED AS ANALYSIS TARGETS - RATIONALE

Many of the Allergens in the SCCS 'Opinion' documentation submitted to the EU Commission consist of multiple isomers of the same molecules and/or complex mixtures.

In many cases, this complexity can be resolved by this Procedure but that leaves a number of target analytes where this isn't the case.

In addition, the SCCS Opinion also documents a number of RN CAS related to the same parent molecule. These are related to the optically active forms of the 'flat' parent structure. For this procedure, the optically active forms are not considered as these require specialist (and in some cases, very different) GC phases to achieve the required separation.

Therefore, this Procedure only addresses the racemic forms of these analytes and optical isomers are not covered as individual analytes.

To enable the rationale behind the selection of each specific analyte and RN CAS covered by this procedure, please refer to **Table 18** in this Appendix for clarification.

Table 18 - Allergens Selected as Analysis Targets - Rationale

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.1	alpha Acetyl cedrene (Vertofix® main)	RN CAS [32388-55-9]	RN CAS [32388-55-9]	The principle isomer (RN CAS [32388-55-9]) only is quantified in this procedure.
3.2.2.	Acetyl isoeugenol / Isoeugenyl acetate	RN CAS [93-29-8]	RN CAS [93-29-8]	
3.2.3	Amyl salicylate (n-amyl salicylate)	RN CAS [2050-08-0]	RN CAS [2050-08-0]	
3.2.4	alpha Amyl cinnamaldehyde (Flosal®)	RN CAS [122-40-7]	RN CAS [78605-96-6]	Only the E isomer (RN CAS [78605-96-6]) is quantified in this procedure.
3.2.5	alpha Amylcinnamyl alcohol	RN CAS [101-85-9]	RN CAS [184900-07-0]	Only the E isomer (RN CAS [184900-07-0]) is quantified in this procedure.
3.2.6	Anethole	RN CAS [4180-23-8]	RN CAS [4180-23-8]	Only the E isomer (RN CAS [4180-23-8]) is quantified in this procedure.
3.2.7	Anise alcohol	RN CAS [105-13-5]	RN CAS [105-13-5]	Para- (4-methoxy) isomer only analysed.
3.2.8	Benzaldehyde	RN CAS [100-52-7]	RN CAS [100-52-7]	
3.2.9	Benzyl alcohol	RN CAS [100-51-6]	RN CAS [100-51-6]	
3.2.10	Benzyl benzoate	RN CAS [120-51-4]	RN CAS [120-51-4]	
3.2.11	Benzyl cinnamate	RN CAS [103-41-3]	RN CAS [103-41-3]	Only the E isomer (RN CAS [103-41-3]) is quantified in this procedure.
3.2.12	Benzyl salicylate	RN CAS [118-58-1]	RN CAS [118-58-1]	

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.13	Camphor	RN CAS [76-22-2] RN CAS [464-49-3]	RN CAS [76-22-2]	RN CAS [464-49-3] relates to the (d-) optical isomer of camphor and is not selectively analysed in this procedure. Only the racemic form RN CAS [76-22-2] is quantified.
3.2.14	Carvone	RN CAS [99-49-0], RN CAS [6485-40-1] RN CAS [2244-16-8]	RN CAS [99-49-0]	RN CAS [6485-40-1] and [2244-16-8] relate to the optical isomers (l-) and (d-) Carvone respectfully and are not selectively analysed in this procedure. Only the racemic form RN CAS [99-49-0] is quantified.
3.2.15	beta Caryophyllene	RN CAS [87-44-5]	RN CAS [87-44-5]	
3.2.16	Cinnamaldehyde	RN CAS [104-55-2]	RN CAS [104-55-2]	Only the E isomer (RN CAS[104-55-2]) is quantified in this procedure.
3.2.17	Cinnamyl alcohol	RN CAS [104-54-1]	RN CAS [104-54-1]	Only the E isomer (RN CAS[104-54-1]) is quantified in this procedure.
3.2.18	Citral	RN CAS [5392-40-5], isomers: RN CAS [106-26-3] RN CAS [141-27-5]	RN CAS [106-26-3] RN CAS [141-27-5]	Citral (RN CAS [5392-40-5] comprises two resolvable components - Neral (Z isomer; RN CAS [106-26-3]) and geranial (E isomer, RN CAS [141-27-5]). Both are quantified and then summed to arrive at reported final Citral concentration in this procedure.

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.19	Citronellol	RN CAS [106-22-9] RN CAS [1117-61-9] RN CAS [7540-51-4]	RN CAS [106-22-9]	RN CAS [1117-61-9] and [7540-51-4] are the optically active 3R (d-) and 3S (l-) forms of Citronellol. These are not selectively analysed in this procedure. Only the racemic form RN CAS [106-22-9] is quantified.
3.2.20	Coumarin	RN CAS [91-64-5]	RN CAS [91-64-5]	
3.2.21	beta Damascenone (Rose Ketone-4)	RN CAS [23696-85-7]	RN CAS [23696-85-7]	
3.2.22	alpha Damascone	RN CAS [43052-87-5]	RN CAS [57549-92-5]	Only the E isomer (RN CAS [57549-92-5]) is quantified in this procedure.
3.2.23	(E) beta Damascone	RN CAS [23726-91-2]	RN CAS [23726-91-2]	
3.2.24	delta Damascone (Rose Ketone-3)	RN CAS [57378-68-4]	RN CAS [71048-82-3]	Only the major isomer - trans/trans delta Damascone (RN CAS [71048-82-3]) is quantified in this procedure.
3.2.25	Dimethylbenzylcarbinyl acetate (DMBCA)	RN CAS [151-05-3]	RN CAS [151-05-3]	
3.2.26	Ebanol	RN CAS [67801-20-1]	RN CAS [67801-20-1]	The procedure measures two components (Ebanol 1; Ebanol 2) which comprise unresolvable, multiple diastereoisomeric pairs. There are no RN CAS which adequately describe these pairs. The sum of these two isomers is used for the final declared values of Ebanol.
3.2.27	Eugenol	RN CAS [97-53-0]	RN CAS [97-53-0]	
3.2.28	Eugenyl acetate	RN CAS [93-28-7]	RN CAS [93-28-7]	

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.29	Farnesol[1]	RN CAS [4602-84-0]	RN CAS [106-28-5]	RN CAS [4602-84-0] refers to generic Farnesol. This comprises four isomers of which the primary (*E,E*)-Farnesol (RN CAS [106-28-5]) is used as the calibration standard for the other isomers if found to be present[1].
3.2.30	Galaxolide (Hexamethylindanopyran)	RN CAS [1222-05-5]	RN CAS [1222-05-5]	Only the two main isomers of Galaxolide (Galaxolide 1 and Galaxolide 2 in the procedure) are quantified as they constitute the greatest composition contribution.
3.3.31	Geraniol	RN CAS [106-24-1]	RN CAS [106-24-1]	
3.3.32	Geranyl acetate	RN CAS [105-87-3]	RN CAS [105-87-3]	
3.3.33	Hexadecanolactone/Dihydroambrettolide	RN CAS [109-29-5]	RN CAS [109-29-5]	
3.3.34	alpha Hexylcinnamaldehyde (Jasmonal®)	RN CAS [101-86-0]	RN CAS [101-86-0]	Only the E isomer (RN CAS [165184-98-5]) is quantified in this procedure.
3.2.35	Hydroxycitronellal	RN CAS [107-75-5]	RN CAS [107-75-5]	
3.2.36	Tetramethylacetyloctahydronaphthalene (ISO E® Super)	RN CAS [68155-66-8] RN CAS [54464-57-2] RN CAS [68155-67-9] RN CAS [54464-59-4]	RN CAS [54464-57-2] RN CAS [68155-66-8] RN CAS [68155-67-9]	Only beta (RN CAS [54464-57-2] - main isomer), alpha (RN CAS [68155-66-8]) and gamma (RN CAS [68155-67-9]) are quantified to represent the total ISO E® Super concentration.

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.37	Isoeugenol	RN CAS [97-54-1]		Only 'E' isomer analysed; the 'Z' isomer being very minor. If seen, the concentration of the 'E' isomer would create a labelling need without the addition of the Z isomer to the total reported.
3.2.38	alpha Isomethylionone	RN CAS [127-51-5]	RN CAS [127-51-5]	Only this specific isomer is analysed.
3.2.39	Butylphenyl methylpropional (Lilial®)	RN CAS [80-54-6]	RN CAS [80-54-6]	
3.2.40	Limonene	RN CAS [5989-27-5]	RN CAS [138-86-3]	RN CAS [5989-27-5] relates to d-limonene and is not resolvable from the l-form by this procedure. Therefore only the racemate RN CAS [138-86-3] is analysed.
3.2.41	Linalool	RN CAS [78-70-6]	RN CAS [78-70-6]	
3.2.42	Linalyl acetate	RN CAS [115-95-7]	RN CAS [115-95-7]	
3.2.43	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (HICC) (Lyral®)	RN CAS [31906-04-4]	RN CAS [31906-04-4] RN CAS [51414-25-6]	The RN CAS quoted refers to the principle isomer of this material (HICC (major) in the procedure). A second isomer (HICC (minor) - RN CAS [51414-25-6]) is also referenced in the procedure as a target analyte. At very low concentrations of the HICC major isomer the minor isomer may not be seen in the final analysis.
3.2.44	Trimethyl-benzenepropanol (Majantol®)	RN CAS [103694-68-4]	RN CAS [103694-68-4]	

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.45	Menthol	RN CAS [1490-04-6] RN CAS [89-78-1] RN CAS [2216-51-5]	RN CAS [89-78-1]	RN CAS [1490-04-6] and [2216-51-5] relate to the optical isomers of menthol. These are not resolvable in this procedure and therefore only the racemic form (RN CAS [89-78-1]) is quantified.
3.2.46	Methyl salicylate	RN CAS [119-36-8]	RN CAS [119-36-8]	
3.2.47	Methyl-2-octynoate (Folione®)	RN CAS [111-12-6]	RN CAS [111-12-6]	Methyl heptane carbonate as an alternative name
3.2.48	alpha Pinene	RN CAS [80-56-8]	RN CAS [80-56-8]	
3.2.49	beta Pinene	RN CAS [127-91-3]	RN CAS [127-91-3]	
3.2.50	3-Propylidene phthalide	RN CAS [17369-59-4] *E*-isomer: RN CAS [56014-72-3] *Z*-isomer: RN CAS [94704-89-9]	*E*-isomer: RN CAS [56014-72-3] *Z*-isomer: RN CAS [94704-89-9]	Both *E* and *Z* isomers are resolvable and therefore included in the procedure; referred to in the documentation as the major' and 'minor' isomers.
3.2.51	Salicylaldehyde	RN CAS [90-02-8]	RN CAS [90-02-8]	
3.2.52	Santalol	RN CAS [11031-45-1]	RN CAS[115-71-9] RN CAS [77-42-9]	The procedure measures two components (alpha Santalol - RN CAS [115-71-9] and beta Santalol - RN CAS [77-42-9]). The RN CAS [11031-45-1] relates to the bulk Santalol.
3.2.53	Sclareol	RN CAS [515-03-7]	RN CAS [515-03-7]	
3.2.54	alpha Terpinene	RN CAS [99-86-5]	RN CAS [99-86-5]	

Reference ID	Analyte	RN CAS as cited in SCCS Opinion and EU Legislative proposal.	Specific RN CAS of Target quantified in Procedure.	Rationale behind discrepancy between SCCS and Target Analyte RN CAS.
3.2.55	Terpineol[2]	RN CAS [98-55-5]	RN CAS [98-55-5]	RN CAS for alpha Terpineol as primary isomer to be quantified by the procedure[2]
3.2.57	Terpinolene	RN CAS [586-62-9]	RN CAS [586-62-9]	
3.2.58	Vanillin	RN CAS [121-33-5],	RN CAS [121-33-5]	

[1] All four Farnesol isomers are present in the calibration mix and these should be used to establish retention times and SIM windows, etc. However, depending on the concentration in the sample, not all of these will be detected by this procedure and the calibration for the lower levels may become compromised. Therefore, for all the isomers of Farnesol, the recommendation is to use the calibration curve for (*E,E)*-Farnesol (RN CAS [106-28-5]) and apply this to the other components to establish the total concentration present.

[2] alpha Terpineol is quantified and the calibration curve for this constituent is used to determine the concentration of the remaining isomers of Terpineol present (cis-beta Terpineol; trans-beta Terpineol; gamma Terpineol). These additional isomers, if found in the analysis, should be summed with the alpha Terpineol value to derive a total Terpineol concentration in the sample.

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