



BSI Standards Publication

**Method for analysis of allergens — Quantification
of an extended list of 57 suspected allergens
in ready to inject fragrance materials by gas
chromatography mass spectrometry**

National foreword

This British Standard is the UK implementation of EN 16274:2021. It supersedes BS EN 16274:2012, which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CW/217, Cosmetics.

A list of organizations represented on this committee can be obtained on request to its committee manager.

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fragrance materials by gas chromatography mass
spectrometry

Méthode d'analyse des allergènes - Quantification de la
liste étendue des 57 allergènes suspectés dans les
matières premières de parfumerie et les compositions
parfumantes prêtes à être injectées, par
chromatographie en phase gazeuse/spectrométrie de
masse

Analyseverfahren für Allergene - Quantifizierung einer
erweiterten Liste von 57 zu vermutenden Allergenen
in einspritzfertigen Duftstoffen mittels
Gaschromatographie/Massenspektrometrie

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European foreword

This document (EN 16274:2021) has been prepared by Technical Committee CEN/TC 347 “Methods for analysis of allergens”, the secretariat of which is held by SNV.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by November 2021, and conflicting national standards shall be withdrawn at the latest by November 2021.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 16274:2012.

The most significant technical changes made in comparison to the previous version are as follows:

- Number of allergens has been extended from 26 to 57 chemically defined molecules.
- Solvents: moves from methyl pivalate and ortho fluorotoluene and acetone to methyl pivalate and MTBE and other solvents provided they are tested prior being used.
- Sample preparation: allows to use weight/volume approach rather than only weight/weight.
- Data processing: provides explanations for a calibration curve using a forced through zero approach.

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Introduction

Directive 2003/15/EC amending 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 in ready to inject fragrance compounds and fragrance raw materials.

The method described in this document does not include requirements for the preparation of samples in matrices for which direct injection in GC is not feasible.

The present document describes a working analytical method based on IFRA Analytical Working Group developments. The analytical method was validated based on a ring test performed by the CEN working group using the accuracy profile approach.

1 Scope

The present method permits the identification and quantification of the volatile compounds suspected as allergens, which are present in the fragrance compounds and fragrance raw materials used in cosmetic products. The analysis is performed by gas chromatography and mass spectrometry (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 1459/11 opinion document (1) 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 found in Annex J.

The method was validated at IFRA and CEN level.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at <http://www.electropedia.org/>
- ISO Online browsing platform: available at <https://www.iso.org/obp>

4 Principle

This procedure has a calibration range between 2 ppm and 240 ppm, this permits the quantification of suspected allergens in diluted matrices in the range 20 ppm to 2 400 ppm per analyte. Beyond the upper concentration level the recommendation is that the sample should be diluted further, or that GC-FID (GC with Flame Ionization Detection) is used preferably in combination with internal standard and response factors.

The matrix samples are analysed for suspected allergens by GC-MS in a total of 4 runs, using 2 analyte sets, both injected on two separate columns of differing polarity. Where necessary the matrix samples should first be diluted in an appropriate solvent.

Their identification and quantification are achieved by selected ion monitoring (SIM; SIM-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’ (see Annex F, Figure F.1) 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 if only SIM methodology was used.

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.

5 Reagents

5.1 Solvents

5.1.1 Methyl pivalate

CAS [598-98-1]

Purity $\geq 99\%$

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 shall be analysed and confirmed as not giving rise to interfering artefacts.

5.1.2 Tert-Butyl Methyl Ether (MTBE)

CAS [1634-04-4]

Purity $\geq 99\%$

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 shall be analysed and confirmed as not giving rise to interfering artefacts.

Alternative solvents are available. If these are used for this method, then the Operator shall undertake sufficient evaluation to verify that the solvent contains no constituents that would interfere with the analytes in this method.

5.2 Reference samples (suspected allergens)

5.2.1 General

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

5.2.1.1 Acetyl cedrene alpha (main isomer in Vertofix® referred to in CAS number), CAS No. [32388-55-9]

Highly variable composition with at least twelve constituents of molecular weight equal to 246 atomic mass units (hereafter u).

5.2.1.2 Acetyl isoeugenol / Isoeugenyl acetate, CAS No. [93-29-8]

5.2.1.3 Amyl salicylate, pentyl salicylate CAS No. [2050-08-0]

This can contain isoamyl salicylate (CAS No. [87-20-7]), which should not be included in this assay.

5.2.1.4 Alpha amyl cinnamaldehyde (Flosal®), CAS No. [122-40-7, 78605-96-6]

2 possible isomers (*E*, *Z*); only *E* is quantified.

5.2.1.5 Alpha amylcinnamyl alcohol, CAS No. [101-85-9, 184900-07-0]

2 possible isomers (*E*, *Z*); only *E* is quantified.

5.2.1.6 Anethole, CAS No. [104-46-1, 4180-23-8]

2 possible isomers (*E*, *Z*); only *E* is quantified.

5.2.1.7 Anise alcohol, CAS No. [105-13-5]

5.2.1.8 Benzaldehyde, CAS No. [100-52-7]

5.2.1.9 Benzyl alcohol, CAS No. [100-51-6]

5.2.1.10 Benzyl benzoate, CAS No. [120-51-4]

5.2.1.11 Benzyl cinnamate, CAS No. [103-41-3]

2 possible isomers (E,Z), only *E* is quantified.

5.2.1.12 Benzyl salicylate, CAS No. [118-58-1]

5.2.1.13 Camphor, CAS No. [76-22-2]

5.2.1.14 Carvone, CAS No. [99-49-0]

5.2.1.15 Caryophyllene beta, CAS No. [87-44-5]

5.2.1.16 Cinnamaldehyde, CAS No. [104-55-2, 14371-10-9]

2 possible isomers (*E*, *Z*), only *E* is quantified.

5.2.1.17 Cinnamyl alcohol, CAS No. [104-54-1, 4407-36-7]

2 possible isomers (*E*, *Z*), only *E* is quantified.

5.2.1.18 Citral, CAS No. [5392-40-5]

5.2.1.18.1 Neral

5.2.1.18.2 Geranial

Both Neral (*Z* isomer, CAS No. [106-26-3]) and Geranial (*E* isomer, CAS No. [141-27-5]) are quantified.

5.2.1.19 Citronellol, CAS No. [106-22-9]

5.2.1.20 Coumarin, CAS No. [91-64-5]

5.2.1.21 Damasconone beta (Rose Ketone-4), CAS No. [23696-85-7]

5.2.1.22 Damascone alpha, CAS No. [43052-87-5] covers both *E* and *Z* isomers

2 possible isomers (*E*, *Z*); only the *E* main isomer CAS No. [24720-09-0] (92 to 99) % is quantified.

5.2.1.23 Damascone beta (*E*), CAS No. [23726-91-2]

5.2.1.24 Damascone delta (Rose Ketone-3), CAS No. [57378-68-4]

3 possible isomers (trans/trans, cis/trans, trans/cis) only the major isomer (trans/trans, up to 90) % is quantified.

5.2.1.25 Dimethylbenzylcarbinyl acetate (DMBCA), CAS No. [151-05-3]

5.2.1.26 Ebanol, CAS No. [67801-20-1]

5.2.1.26.1 Ebanol Isomer 1

5.2.1.26.2 Ebanol Isomer 2

The 2 isomers (*E*, *Z*) are quantified.

5.2.1.27 Eugenol, CAS No. [97-53-0]**5.2.1.28 Eugenyl acetate, CAS No. [93-28-7]****5.2.1.29 Farnesol, CAS No. [4602-84-0]**

Recommendation: Use E,E calibration curve to quantify other isomers.

4 possible isomers: (*E,E*) isomer is CAS No. [106-28-5] and the (*Z,E*) isomer is CAS No. [3790-71-4], and two additional minor isomers.

5.2.1.30 Galaxolide (Hexamethylindanopyran), CAS No. [1222-05-5]

Only the two main isomers are quantified.

5.2.1.31 Geraniol, CAS No. [106-24-1]**5.2.1.32 Geranyl acetate, CAS No. [105-87-3]****5.2.1.33 Hexadecanolactone / Dihydroambrettolide, CAS No. [109-29-5]****5.2.1.34 Hexylcinnamaldehyde alpha, CAS No. [101-86-0, 165184-98-5]**

2 isomers at least; only the *E* is quantified.

5.2.1.35 Hydroxycitronellal, CAS No. [107-75-5]**5.2.1.36 Tetramethylacetyloctahydronaphthalene (ISO E® Super)**

NOTE This consists of several isomers including the alpha, (CAS No. [68155-66-8]); the major isomer, beta (CAS No. [54464-57-2]); the gamma (CAS No. [68155-67-9]) and a minor (CAS No. [54464-59-4]).

5.2.1.37 Isoeugenol, CAS No. [97-54-1]

2 possible isomers (*E*, *Z*), only *E*- isoeugenol is quantified.

5.2.1.38 Isomethylionone alpha, CAS No. [127-51-5]

Can contain beta isomethylionone (CAS No. [79-89-0]), alpha methylionone (CAS No. [7779-30-8]), beta methylionone (CAS No. [127-43-5]), pseudo isomethylionone (CAS No. [1117-41-5]).

Other grades may contain other isomers including the major one. These constituents should not be assayed.

5.2.1.39 Butylphenyl methylpropional (Lilial®), CAS No. [80-54-6]**5.2.1.40 Limonene, CAS No. [138-86-3]****5.2.1.41 Linalool, CAS No. [78-70-6]****5.2.1.42 Linalyl acetate, CAS No. [115-95-7]**

5.2.1.43 Hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyrall®), CAS No. [31906-04-4]

Contains Hydroxyisohexyl 4-cyclohexene carboxaldehyde (CAS No. 51414-25-6) (noted Lyrall minor) that has to be quantified.

5.2.1.44 Trimethyl-benzenepropanol (Majantol®), CAS No. [103694-68-4]

5.2.1.45 Menthol, CAS No. [1490-04-6, 89-78-1]

5.2.1.46 Methyl salicylate, CAS No. [119-36-8]

5.2.1.47 Methyl-2-octynoate (Folione®), CAS No. [111-12-6]

5.2.1.48 Pinene alpha, CAS No. [80-56-8]

5.2.1.49 Pinene beta, CAS No. [127-91-3]

5.2.1.50 3-Propylidene phthalide, CAS No. [17369-59-4]

The 2 isomers (*E,Z*) (*E*-isomer: CAS No. [56014-72-3] and *Z*-isomer: CAS No. [94704-89-9]) are quantified.

5.2.1.51 Salicylaldehyde, CAS No. [90-02-8]

5.2.1.52 Santalol, CAS No. [11031-45-1]

NOTE The 2 isomers, alpha-santalol (CAS No. [115-71-9]) and beta-santalol (CAS No. [77-42-9]), are quantified.

5.2.1.53 Sclareol, CAS No. [515-03-7]

5.2.1.54 Terpinene alpha, CAS No. [99-86-5]

5.2.1.55 Terpineol-alpha, CAS No. [98-55-5]

5.2.1.55.1 for terpineol gamma CAS No. [586-81-2]

5.2.1.55.2 for Terpineol *cis*-beta CAS No. [138-87-4]

5.2.1.55.3 for Terpineol *trans*-beta CAS No. [7299-41-4]

Recommendation: use alpha-terpineol calibration curve to quantify other isomers.

5.2.1.56 Terpinolene, CAS No. [586-62-9]

5.2.1.57 Vanillin, CAS No. [121-33-5]

5.3 Internal standards (ISTD)

5.3.1 1,4-Dibromobenzene (IS_A), CAS No. [106-37-6], purity ≥ 98 %

5.3.2 4,4'-dibromobiphenyl (IS_B), CAS No. [92-86-4], purity ≥ 98 %

6 Apparatus

6.1 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 (above 2,4 % as found in essential oils for example). It is recommended to perform the purity study using the procedure described in Annex A.

A GC-FID method is available [3] for the measurement of analytes at concentrations above 2,4 %. 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.

6.2 Gas chromatograph coupled to a mass spectrometer (GC-MS)

6.2.1 General

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

6.2.2 GC-MS system

- a) Equipped with electronic control of carrier gas pressures and/or flows;
- b) it is recommended that an autosampler, fitted with a syringe of suitable size, be used for the injection of the calibration and sample solutions;
- c) the glass injection liner shall be inert with an interior volume compatible with the expansion volume of the dilution solvent;
- d) two capillary columns of different phase types are to be used for quantitative analysis;
- e) mass spectrometer tuning and the levels of air and water should be checked regularly to ensure optimal sensitivity, as well as the injector cleanliness to maintain analytical performance.

The sensitivity of the mass spectrometer should also be optimized through maximizing the detector signal to noise ratio. One option could be to increase the detector (electron multiplier) voltage according to the instrument capabilities and manufacturers recommendations.

6.3 Capillary columns for GC

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

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 m or 60 m). 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) and validate that length for the analyte(s) being investigated (see Annex B).

Table 1 — Recommended GC Column Phase/Types and Analytical Parameters

Phase	Dimensions (Length × internal diameter × thic kness)	Recommended oven temperature program	Injection conditions	Carrier gas	MS conditions	Mean resolution $\overline{R}$	
100 % Polydimethylsiloxane (PDMS)	30 m × 0,25 mm × 0,25 μm	80 °C for 4 min, then 15 °C/min to 105 °C for 2 min, then 4 °C/min to 150 °C, then 10 °C/min to 280 °C – hold 5 min.	Split/ splitless injector at 250 °C - Deactivated liner- Inject 1 μL with split 1:20 – Inj. Temp. 250 °C (same for both calibration and samples)	Helium Constant flow at 1,2 mL/m in (velocity approx.. 40 cm/se c)	Source Temperature at 250 °C – Quadrupole temp. at 150 °C – Scanning mass range (m/z) from 35 Da to 350 Da	SIM method 1 1.43	
					-	SIM method 2 1.40	
50 % Phenylmethylpolysiloxane 50 % Polydimethylsiloxane (PDMS)	30 m × 0,25 mm × 0,25 μm	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 – hold 5 min			-	Ionization energy: 70 eV	SIM method 1 1.40
					-	SIM method 2 1.42	

6.4 Analytical balance

All weighing of reference samples and internal standards shall be carried out on an analytical balance with 0,000 1 g readability.

7 Stock and sample solutions - preparation and storage

7.1 General information

7.1.1 Choice of the solvent

Methyl pivalate and MTBE are suitable for the preparation of stock and calibration solutions as well as the dilution of samples prior to analysis as they have been proven to satisfy the following requirements:

- 1) inertness to components and allergens in fragrance matrices;
- 2) medium volatility to ensure solution's stability and concentration;
- 3) expansion volume compatible with the interior volume of the injector insert.

Recommendation: For information related to the solvent, see 5.1.

The use of any other solvent requires the user to perform preliminary tests to demonstrate, in particular, inertness towards the analytes and to determine its purity. The use of solvents possessing hydroxyl or carbonyl functions (ethanol, methanol, acetone...) is not recommended in order to avoid degradation or reaction with the analytes (e.g. acetal formation).

7.1.2 Miscellaneous

The same solvent shall be used to prepare the calibration solutions, the system blank and to dilute samples.

7.2 Preparation of stock solutions from reference samples

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 Annex G.

The following description suggests working using a weight/weight approach when preparing the different solutions. It is also possible to use volumes when preparing the diluted solutions without any drastic loss of accuracy in the final results.

7.3 Preparation of internal standard solutions and calibration solutions

7.3.1 General

All below explanations are provided for a weight/weight approach. In case of weight/volume, the only difference is that the analyst does not have to weigh the solvent but rather make up with the solvent to the mark in the flask. Operators can refer to Annex G if externally purchased solutions are not used.

7.3.2 Stock Solutions of Internal Standards (1g/kg equivalent)

Prepare a stock solution (S-ISTD) containing 1 g of 1,4-dibromobenzene (5.3.1) and 1 g 4,4'-dibromobiphenyl (5.3.2) made up to 1 kg with methyl pivalate or MTBE. This solution can be prepared as follows:

- Prepare a solution (S-ISTD) by weighing accurately 0,1 g of each compound (5.3.1 and 5.3.2) in a suitable flask and make up to 100 g with methyl pivalate or MTBE.
- In case of weight/weight concentrations, note the weight of each compound and the final weight of solvent used, to determine the final solution concentration of each internal standard.
- Pay attention to the differences in density between the solvents. For instance, for MTBE, relative density is 0,740 4, thus only weigh 74 mg of each of the standards and complete with MTBE in a 100 ml flask (the volume of added MTBE will be 74 g).

7.3.3 Calibration Solutions Concentrations

To limit chemical reactions between, and degradation of analytes, 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.

These 2 solutions are called A1 and A2 respectively.

The calibration solutions are prepared from these two sets of 'stock' solutions, A1 and A2, containing the analytes at a nominal concentration of 2 g/kg. The final calibration solutions are arrived at by a dilution of these solutions (or a further derivative of these; A3 and A4) to a known weight with methyl pivalate or MTBE.

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.

To avoid dispensing very small (i.e. < 10 µL) 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 (Table 2 and Table 3).

Note the weight of all standard solutions as well as the solvent weight, in case you are using w/w concentrations.

Table 2 — Preparation volume for Solution A3

Primary Solution (2 g/kg)	Volume of Primary Solution	Final Volume of Diluted Solution (A3)	Final Concentration of Allergens (mg/kg)
A1	1 mL	10 mL	200

Table 3 — Preparation volume for Solution A4

Primary Solution (2 g/kg)	Volume of Primary Solution	Final Volume of Diluted Solution (A4)	Final Concentration of Allergens (mg/kg)
A2	1 mL	10 mL	200

Ensure they are adequately stoppered to minimize any potential evaporation of the solvent.

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. One month stability period was ascertained under these conditions for the two stock solutions A1 and A2 as well as for A3 and A4.

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

7.3.4 Preparation of Calibration solutions

Prepare the calibration solutions C1, C2, C3 by diluting the two allergen primary solutions, A1 and A2 to 10 mL with methyl pivalate or MTBE after the addition of the internal standard solution, (S-ISTD), (8.3) according to the following instructions, and referring to the dilution scheme in Table 4. The weight of the individual combined solutions shall be recorded.

Similarly, prepare the calibration solutions C4, C5, C6, C7 by diluting solutions A3 and A4 (8.3) to 10 mL with methyl pivalate after the addition of the internal standard solution, (S-ISTD), (8.3) according to the dilution scheme in Table 4. The weight of the individual combined solutions shall be recorded.

To create the Calibration Solutions C1 through C3, the following process should be adopted:

- For Solution C1: Dispense 1,2 mL of Solution A1 and 1,2 mL of solution A2 into a 10 mL tared volumetric flask, noting the weights of A1 and A2 (m_{A1} and m_{A2} respectively) taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A1} + m_{A2} + m_{ISTD} + m_{solvent}$.

- For Solution C2: Dispense 0,6 mL of Solution A1 and 0,6 mL of solution A2 into a 10 mL tared volumetric flask, noting the weights of A1 and A2 (m_{A1} and m_{A2} respectively) taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A1} + m_{A2} + m_{ISTD} + m_{solvent}$.

- For Solution C3: Dispense 0,25 mL of Solution A1 and 0,25 mL of solution A2 into a 10 mL tared volumetric flask, noting the weights of A1 and A2 (m_{A1} and m_{A2} respectively) taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A1} + m_{A2} + m_{ISTD} + m_{solvent}$.

For Solutions C4 to C7, the solutions A3 and A4 should be used to avoid potential errors in dispensing small weights of A1 and A2:

- For Solution C4: Dispense 1 mL of Solution A3 and 1 mL of solution A4 into a 10 mL tared volumetric flask, noting the weights of A3 and A4 taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A3} + m_{A4} + m_{ISTD} + m_{solvent}$.

- For Solution C5: Dispense 0,5 mL of Solution A3 and 0,5 mL of solution A4 into a 10 mL tared volumetric flask, noting the weights of A3 and A4 taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A3} + m_{A4} + m_{ISTD} + m_{solvent}$.

- For Solution C6: Dispense 0,25 mL of Solution A3 and 0,25 mL of solution A4 into a 10 mL tared volumetric flask, noting the weights of A3 and A4 taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A3} + m_{A4} + m_{ISTD} + m_{solvent}$.

- For Solution C7: Dispense 0,1 mL of Solution A3 and 0,1 mL of solution A4 into a 10 mL tared volumetric flask, noting the weights of A3 and A4 taken at each step.

Add 1 mL of the Internal Standard solution, note the weight, and make up to the 10 mL mark with solvent.

Note the final weight = $m_{A3} + m_{A4} + m_{ISTD} + m_{solvent}$.

The 7 points calibration curve has been used to validate this method. It is also possible to work with a 6 point calibration curve keeping only C1(240 mg/kg), C2 (120 mg/kg), C3 (50 mg/kg), C4 (20 mg/kg), C5 (10 mg/kg), C7 (2 mg/kg).

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

Table 4 — Individual Allergen Calibration Standard Solution concentrations

	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 (2 g/kg)	A2 (2 g/kg)	(1 g/kg)		
C1	240	1,200	1,200	1,000	100	10
C2	120	0,600	0,600	1,000	100	10
C3	50	0,250	0,250	1,000	100	10
Secondary Solutions		A3 (0,2 g/kg)	A4 (0,2 g/kg)	(1 g/kg)		
C4	20	1,000	1,000	1,000	100	10
C5	10	0,500	0,500	1,000	100	10
C6	5	0,250	0,250	1,000	100	10
C7	2	0,100	0,100	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 h.

8 Mass spectrometer acquisition conditions

8.1 Establishment of retention times and SIM chromatograph 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.

It is recommended that a concentration in the mid-range of the calibration curve be selected.

For each column, the user will create two SIM or SIM-SCAN methods (SIM1 and SIM2) in order to analyse the 57 potentially allergenic substances in two sets of analytes.

Typical SIM ions (I1, I2 and I3) and their associated Internal Standard are detailed in Table C.1, Table C.2, Table C.3 as given in Annex C. 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.

8.2 SIM Window – Set up and criteria

The choice of internal standard to be assigned for quantification of each analyte is also provided in Annex C, Table C.1, Table C.2 and Table C.3.

Examples of SIM windows for the two sets of analytes and the two columns are given in Annex D, Table D.1, Table D.2, Table D.3 and Table D.4.

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 may contain up to 9 or more ions. In each case, the dwell time should be chosen to have a minimum of 3 scan cycles per sec, and/or at least 10 to 15 acquisition points per chromatographic peak for accurate integration.

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

8.3 GC-MS scan mode verification

A full SCAN mode is required to identify the presence of peaks that are thought to have moved out of their usual SIM window or been hidden by interfering peaks (see Annex E, including Figures E.1, E.2, E.3 and E.4).

For complementary information, see Annex K.

9 Sample analysis

9.1 General

It is recommended to perform a 10-fold dilution of the sample prior to analysis, which implies weighing exactly about 1 g of sample in a 10 mL volumetric flask. If analyte concentrations are found to be outside of the calibration range, then dilution of another sample up to a maximum dilution of 240-fold can be undertaken with re-analysis.

9.2 Sample preparation for analysis

- Accurately weigh (to the nearest milligram) a sample amount (typically 1 g), in a 10 mL volumetric flask.
- Add 1,0 mL of the internal standard solution (S-ISTD), 8.3, and note the weight. The final concentration of internal standard added should be the same as that used in the calibration solutions.
- Adjust to the 10 mL mark with methyl pivalate (or the same dilution solvent as that used to prepare calibration solutions).
- Note the final weight (mDIL) of this solution.

Homogenize and transfer an aliquot of the prepared sample into a GC vial for GC-MS analysis.

9.3 Sequence

9.3.1 Blank

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.

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 above 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.

9.3.2 Calibration solutions

Inject each Calibration solution (C1 through C7) on the two separate GC-MS columns according to the SIM or SIM-SCAN methods.

For each allergen, each ion in Table C.1 and Table C.2 (Annex C) (e.g. I1) is used in turn as the quantifier ion, with the remaining two ions in Table C.3 (e.g. I2 and I3) being used as the Qualifier ions.

One calibration per column type and per ion 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 R^2 shall be above 0,995 and the residues shall be distributed on either side of the curve.

The recommended calibration curve to apply is:

Quadratic Fit: Force through zero

In addition, a Weighting of $1/\text{Concentration}$ 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 Annex H.

9.3.3 Check solution

The calibration check solution (for instance 20 mg/kg or 20 mg/L) 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.

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

9.3.4 Samples

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).

This should be performed within a 48 h window following sample preparation.

Following the GC-MS analysis of the samples, check the concentration of found analytes, which shall 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 shall be carried out before subsequent GC-MS re-analysis. If this is found to be the case, then a fresh sample shall be prepared from the original sample with a greater dilution factor, i.e. 1 g in 100 mL or equivalent.

It is important to note that over-dilution of the sample can lead to discrimination of higher Molecular Weight constituents in the injection process and, therefore, will lead to skewed results. If a sample dilution of greater than 240-fold is required it is recommended that the analysis for that constituent is performed by GC-FID.

Injection can be done only once on each vial.

10 Data validation and treatment

10.1 General

There are three approaches to data validation:

- use of Q values generated by some vendors' software as part of the analytical report;
- use of corresponding SIM data to compare ion ratios;
- use of scan data for ion ratios which is independent of the quantitation process.

These three approaches are all based on the area ratio determination of the ions and are dedicated to compound identification and confirmation. The Q value and SIM data are derived from peak area measurements whereas scan data provides direct ion abundances from which the ratios are calculated.

For quantification, refer to Annex I.

10.2 Examination of Q values

The reported results from the GC-MS analysis using SIM can be initially evaluated by monitoring the Q value (see Annex F). 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:

$$Q = 100 - \frac{\sum_{i=1}^{i=n} \left(100 \times \left| r_i - r_i^1 \right| \right) \left(\ln [100r_i + 1] \right)^2}{21,3 \times \sum_{i=1}^{i=n} r_i} \quad (1)$$

where

n is the number of qualifier ions for each analyte;

r_i is the reference area ratio; and

r_i^1 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 at a concentration in the mid-range of the calibration curve.

10.3 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 Table 5. This can be conducted by examination of the Q values.

The Table 5 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.

10.4 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 %

10.5 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 Annex 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.

10.6 GC-MS scan mode verification

A full SCAN mode is required to clarify the presence of peaks that are thought to have moved out of their usual SIM window or been hidden by interfering peaks.

10.7 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.

11 Test report

The test report shall contain at least the following information:

- all information necessary for the identification of the sample;
- a reference to this document;
- date and type of sampling (if known);
- date of receipt of the laboratory sample;
- date of test;
- the results and the units in which the results have been expressed;
- any particular points observed in the course of the test;
- any operations not specified in the method or regarded as optional which might have affected the results.