SLOVENSKI STANDARD
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Analizne metode za alergene - Kvantitativno določevanje 57 domnevnih alergenov z razširjenega seznama v dišavnih izdelkih s plinsko kromatografsko analizo vzorcev, ki so pripravljeni za injeciranje

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

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

Méthodes d'analyse des allergènes - Quantification des fragrances allergènes suspectées dans les produits de consommation - Etape 1 : Analyse par GC d'échantillons prêts à être injectés

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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

Méthodes d'analyse des allergènes - Quantification des fragrances allergènes suspectées dans les produits de consommation - Étape 1 : Analyse par GC d'échantillons prêts à être injectés

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This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 347.

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European foreword

This document (prEN 16274:2020) has been prepared by Technical Committee CEN/TC 347 “Methods for analysis of allergens”, the secretariat of which is held by SNV.

This document is currently submitted to the CEN Enquiry.

This document will supersede EN 16274:2012.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

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 raw materials and oils and dilutions thereof.

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 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 with two solvents, namely methyl pivalate and tert-methyl butyl ether.

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 CAS Numbers related to the same parent molecule. These are related to the optically active forms of the similar parent structure. For this procedure, the optically active forms are not considered as these require special (and in some cases, very different) GC phases to achieve the required separation.

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 http://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 Ionisation 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) 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.

This Procedure is written as a methodology to be applied in a working laboratory (QC and R&D type). Particular attention shall be paid by the user to the details contained in the Annexes and to specific comments ('Notes') in the text regarding the application of this methodology.

5 Reagents

5.1 Solvents

5.1.1 Methyl pivalate

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 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 ≥ 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)

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

NOTE Highly variable composition with at least twelve constituents of molecular weight equal to 246 amu.

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

5.2.3 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.

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

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

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

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

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

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

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

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

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

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

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

NOTE Only *E* is quantified.

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

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

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

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

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

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

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

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

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

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

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

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

5.2.21 Damascenone beta (Rose Ketone-4), n° CAS [23696-85-7]

5.2.22 Damascone alpha, CAS No. [43052-87-5]

NOTE 2 possible isomers (*E, Z*); only the *E* main isomer (92 to 99%) is quantified.

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

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

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

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

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

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

5.2.27 Eugenol, CAS No. [97-53-0]

5.2.28 Eugenyl acetate, CAS No. [93-28-7]

5.2.29 Farnesol, CAS No. [4602-84-0]

NOTE 4 possible isomers: (*E,E*) isomer is (n° CAS [106-28-5]) and the (Z,E) isomer is (n° CAS [3790-71-4]).

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

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

NOTE Only the two main isomers are quantified.

5.2.31 Geraniol, CAS No. [106-24-1]

5.2.32 Geranyl acetate, CAS No. [105-87-3]

5.2.33 Hexadecanolactone / Dihydroambrettolide, CAS No. [109-29-5]

5.2.34 Hexylcinnamaldehyde alpha, CAS No. [101-86-0, 165184-98-5]

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

5.2.35 Hydroxycitronellal, CAS No. [107-75-5]

5.2.36 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]).

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

NOTE 2 possible isomers (E, Z), only E- iso eugenol is quantified.

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

NOTE 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. These constituents should not be assayed.

5.2.39 Butylphenyl methylpropional (Lilial®), CAS No. [80-54-6]

5.2.40 Limonene, CAS No. [138-86-3]

5.2.41 Linalool, CAS No. [78-70-6]

5.2.42 Linalyl acetate, CAS No. [115-95-7]

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

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

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

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

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

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

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

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

5.2.50 3-Propylidene phthalide, CAS No. [17369-59-4]

NOTE The 2 isomers (*E,Z*) (*E*-isomer: CAS No.56014-72-3 and Z- isomer: CAS No.94704-89-9) are quantified (isomers ratio near 1:6).

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

5.2.52 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.

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

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

5.2.55 Terpineol-alpha, CAS No. [98-55-5] for alpha-terpineol; 5.2.55a for gamma-terpineol CAS No. [586-81-2]; 5.2.55b for Terpineol *cis*-beta CAS No. [138-87-4]; 5.2.55c for Terpineol *trans*-beta CAS No. [7299-41-4]

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

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

5.2.57 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), v [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 (>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 >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 requirements:

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 weekly, as well as the injector cleanliness to maintain analytical performance.

The sensitivity of the mass spectrometer should also be optimized through maximising 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.

NOTE 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 (see Annex B).

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

Phase[a]	Dimensions (Length × internal diameter × thickness)	Recommended oven temperature program	Injection Conditions	Carrier Gaz	MS conditions	Mean Resolution $\overline{R}$
100% Polydimethylsiloxane	30m × 0.25mm × 0.25µm	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 ul with split 1:20 – Inj. Temp. 250°C (same for both calibration and samples)	Helium Constant flow at 1,2 mL/min (velocity approx.. 40 cm/sec)	Source Temperature at 250 °C – Quadrupole temp. at 150 °C – Scanning mass range (m/z) from 35 to 350 Da - Ionisation energy: 70 eV	SIM method 1 1.43
						SIM method 2 1.40
50% Phenylmethylpolysiloxane 50% Polydimethylsiloxane	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 – hold 5 min.				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 0,0001 g readability

7 Mass Spectrometer Acquisition Conditions

7.1 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

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 and Table C.4 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.

7.2 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, Table C.3 and Table C.4.

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/sec, and/or at least 10 to 15 scans 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.

7.3 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).

For complementary information, see Annex K.

8 Stock and Sample solutions - preparation and storage

8.1 General information

8.1.1 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).

8.1.2 Miscellaneous

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