Validity of a GC and GC-MS method for the quantification of potential contact allergens in cosmetics containing plant extracts and/or essential oils
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Christensen, Lars Porskjaer

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Validity of a GC and GC–MS method for the quantification of potential contact allergens in cosmetics containing plant extracts and/or essential oils

Lars P. Christensen, Danish Institute of Agricultural Sciences, Department of Horticulture, Research Center Aarslev

Contents

Preface
1 Summary and conclusions
  1.1 Summary
  1.2 Conclusions
2 Background
3 Purpose
4 Validity, reference and responsibility
  4.1 Validity
  4.2 Reference
  4.3 Responsibility
5 Method description
  5.1 Demarcation
  5.2 Equipment, chemicals and materials
    5.2.1 Capillary gas chromatography (GC)
    5.2.2 Capillary gas chromatography–mass spectrometry (GC–MS)
    5.2.3 Chemicals and materials
    5.2.4 Investigated cosmetics
  5.3 Preparation of GC–MS equipment
  5.4 Preparation of GC equipment
  5.5 Preparation of samples for GC and GC–MS analysis
    5.5.1 Preparation of external standard solutions
    5.5.2 Preparation of a standard solution containing potential contact allergens
    5.5.3 Preparation of sample solutions of cosmetics containing plant extracts and/or essential oils
  5.6 Identification of potential allergens and/or irritants in cosmetics containing plant extracts and/or essential oils
6 Approval of the analysis
  6.1 Approval of the sample analysed
  6.2 Approval, registration and filing the result
7 Validity of the GC and GC–MS method
  7.1 Robustness
    7.1.1 Column
    7.1.2 Flow
    7.1.3 Temperature
7.2 Detection and quantification limits
7.3 Linearity
7.4 Accuracy
7.5 Precision (repeatability)
8 Concentration of contact allergens in cosmetics
9 References

Preface
Although many fragrance ingredients are now made synthetically the use of essential oils containing a wide spectrum of fragrances are used in large scale in perfumes, creams and in products used in aromatherapy. Essential oils contain different types of natural products of which some may cause allergic contact dermatitis and/or irritative skin reactions and hence adverse skin reactions are frequently reported from cosmetics based on fragrances. Fragrances account for a major part of allergic contact reactions to cosmetics. Approximately one third of investigated cases of cosmetic allergy are identified as being due to fragrances.

A sensitive, reliable and relatively simple method for the detection and quantification of potential contact allergens and/or irritants derived from plant sources in cosmetics is therefore important in order to be able to determine the amounts and distribution of these constituents in cosmetics. Secondly, such a method maybe useful in the search for new contact allergens.

The aim of the project was, thus, to develop a method for extraction, identification and quantification of potential contact allergens and/or irritants in cosmetics containing plant extracts and/or essential oils and finally to determine the validity of the method according to EU guidelines (ICH guidelines).

The project is carried out by the Danish Institute of Agricultural Sciences, Department of Horticulture, Research Center Aarslev.

1 Summary and conclusions
1.1 Summary
Potential contact allergens and/or irritants in cosmetics are often natural products with a molecular weight below 300 amu. A sensitive, reliable and relatively simple method for the detection and quantification of potential contact allergens and/or irritants derived from plant sources in cosmetics was developed. The method is based on analytical techniques such as gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) and validated according to the ICH guidelines from the European Commission (Volume 3A Guidelines, 1998).

Cosmetics containing plant extracts and/or essential oils were investigated for common potential contact allergens and/or irritants. Potential contact allergens and/or irritants that consisted of monoterpenes (e.g., α-pinene, α-terpi-nene, linalyl acetate and linalool) and phenols (eugenol and iso-eugenol) were found in 6 out of 10 investigated cosmetics.

1.2 Conclusions
The following conclusions can be drawn upon the validation of the GC and GC–MS method (DIAS ALLERGENS 1, date 21.12 2001) for the detection and quantification of naturally occurring contact allergens and/or irritants in cosmetics containing plant extracts and/or essential oils.

The method gives a relatively good separation of the selected potential contact allergens and/or irritants, which makes it possible to perform a reliable quantification of the
compounds in commercial product samples. The relatively high degree of linearity, accuracy, precision (repeatability) and robustness of the method further ensures a reliable quantification. The quantification limits of the selected compounds was around 0.0002 mg/ml for the majority of the compounds. Potential contact allergens and/or irritants in cosmetics near the detection and quantification limits of this method are not expected to have any larger impact on contact allergy and/or irritant skin reactions.

2 Background

Products applied to the skin, for non-medicinal reasons, are variously known by terms such as cosmetics or skin care preparations. Plants and substances derived from them have been used in cosmetics since historical times. Throughout the centuries the pleasant smells of many plants have been used to make fragrances for personal application. Although many of these fragrance ingredients are now made synthetically the use of essential oils containing a wide spectrum of these fragrances are used in large scale in perfumes, creams and in products used in aromatherapy. With regard to plant extracts there is often no functional rationale for the inclusion of these in cosmetics. They are in general added for marketing reasons so that claims such as ‘with plant extracts’, ‘with herbal extracts’ or ‘with beneficial oils’ can be made to nurture consumer interest in the perceived benefits of ‘natural ingredients’ on the skin. Some cosmetics may contain significant amounts of plant extracts but most, however, contain trivial amounts, often 0.1% of commercial aqueous extracts, so that a valid claim for their inclusion can be made. Some performance cosmetics contain large numbers of plant extracts, which are marketed with a high and expensive media profile. An exhaustive list of such extracts has been published (Nater & de Groot, 1985).

Plant extracts and/or essential oils contain different types of natural products of which some may cause allergic contact dermatitis and/or irritative skin reactions. Plant extracts from Compositae species are often used in cosmetics, and these extracts may have a high content of highly sensitizing sesquiterpene lactones, e.g., costunolide and parthenolide (Evans & Schmidt, 1980; Hausen, 1988; Lovell, 1993). Also less sensitizing natural products are common constituents in cosmetics including common aroma compounds such as eugenol, isoeugenol, geraniol, carvone and limonene. These compounds are often present in extracts and/or oils from plants that do not belong to the Compositae plant family. Well-known examples are oils from citrus fruits (bergamot oil), jasmine (Jasminum spp.), mint (Mentha spp.) and lavender (Lavendula spp.) (Evans & Schmidt, 1980; Hausen, 1988; Lovell, 1993; Nater & de Groot, 1985).

Despite the huge quantities of plant extracts used by the cosmetic industry, adverse reactions to them among consumers are relatively rare. This rarity of reactions is probably a manifestation of the low concentrations present in the finished product. Adverse skin reactions have been more frequently reported from cosmetics based on fragrances such as fine perfumes and essential oils used in aromatherapy. Fragrances account for a major part of allergic contact reactions to cosmetics. Approximately one third of investigated cases of cosmetic allergy are identified as being due to fragrances (Lovell, 1993). To detect fragrance sensitivity patch testing with the fragrance mix, colophony and balsam of Peru are used as standard indicators for fragrance sensitivity. These indicators may detect fragrance sensitivity in about 70–80% of cases. The fragrance mix consist for example of some common potential fragrance allergens such as eugenol, isoeugenol, cinnamyl alcohol and geraniol (Lovell, 1993).

A sensitive, reliable and relatively simple method for the detection and quantification of potential allergens and/or irritants derived from plant sources in cosmetics is therefore important in order to be able to determine the amounts and distribution of these constituents...
in cosmetics. Secondly, the method maybe useful in the search for new contact allergens.

3 Purpose
The purpose of this instruction is to describe a qualitative and quantitative GC (= gas chromatography) and GC–MS (= gas chromatography–mass spectrometry) method for the analysis of potential contact allergens and/or irritants in cosmetics containing plant extracts and/or essential oils and to determine the validity of the method, named DIAS ALLERGENS 1, date 21.12 2001.

4 Validity, reference and responsibility
4.1 Validity
This instruction is valid for the qualitative and quantitative GC and GC–MS method for the analysis of naturally occurring contact allergens and/or irritants (see Figure 1) in (i) cosmetics containing plant extracts and/or essential oils and (ii) oils used in e.g. aromatherapy (see section 5) at the Danish Institute of Agricultural Sciences (DIAS), Department of Horticulture, Research Group for Food Quality and Natural Products Chemistry, Research Center Aarslev. The GC and GC–MS method described is intended for routine identification and quantification of compounds 1–18 (Figure 1) and structurally related naturally occurring allergens and/or irritants. Information about the allergenic and/or irritant properties of 1–18 and related compounds can be found in Evans & Schmidt (1980), Hausen (1988), Hausen et al. (1999), Lovell (1993) and Mitchell & Rook (1979).

4.2 Reference
The quality handbook for DIAS GC No. 3, DIAS GC–MS No. 1 and DIAS capillary GC columns (DIAS No. 23 and 26).

4.3 Responsibility
The operator of this instruction is responsible for the analysis being performed as specified with an adequate accuracy and precision, including informing the laboratory manager (LM) in case of irregularities. The LM is notified if irregularities occurs during the analysis.
Figure 1. Chemical structures of the contact allergens and/or irritants (1–18) investigated by the method DIAS ALLERGENS 1, date 21.12 2001.

5 Method description
5.1 Demarcation
This method is a GC and GC–MS method for the qualitative and quantitative determination of potential allergens and/or irritants, with a molecular weight below 300 amu, in plant extracts and essential oils, and in cosmetics containing plant extracts and/or essential oils by the use of external standards.
5.2 Equipment, chemicals and materials

5.2.1 Capillary gas chromatography (GC)
GC is primarily used for the quantification of allergens/irritants. GC analysis is performed on a Hewlett-Packard 5890 Series II Plus chromatograph (Hewlett-Packard, Wilmington, USA) equipped with:

- Hewlett-Packard Auto Injector (GC System Injector).
- Flame Ionization Detector (FID).
- A WCOT (Wall Coated Open Tubular) fused capillary column (50 m ´ 0.25 mm i.d., DF = 0.2 mm liquid phase: CP–Wax 52 CB, Cat. No. 7723 (DIAS No. 18), Middleburg, Holland).

The instruments are controlled by PC equipped with a Hewlett-Packard (HP) ChemStation.

5.2.2 Capillary gas chromatography–mass spectrometry (GC–MS)
GC–MS is primarily used for identification and final verification of allergens/irritants. GC–MS is performed on a Varian Saturn 2000 Ion Trap Mass Spectrometer at an ionization potential of 70 eV and interfaced to a Varian Star 3400 CX gas chromatograph (Varian Chromatography Systems, California, USA) equipped with a Rtx-20 capillary column (30 m ´ 0.25 mm i.d., DF = 0.25 mm liquid phase: Cat. no. 10323 (DIAS No. 23), Restek Corporation, Bellefonte, PA, USA).

The instruments are controlled by PC equipped with a Saturn GC–MS Workstation, version 5.2.1.

5.2.3 Chemicals and materials
- Double-distilled dichloromethane (CH$_2$Cl$_2$) (Super Purity Solvent, Romel Ltd, Chr. Gerner-Jensen).
- Compounds 1–17 were obtained from Aldrich (Steinheim, Germany) or Fluka Chemie AG (Buchs, Switzerland). Parthenolide (18) was isolated from feverfew (Tanacetum parthenium (L.) Sch.-Bip.) by column chromatography and preparative TLC as described previously (Christensen et al., 1999; Hausen, 1991) and identified by mass spectrometry and NMR spectroscopy.
- Nitrogen (purified through charcoal filter).
- Helium (purified through charcoal filter).
- Hydrogen.
- Volumetric flasks (5 ml).
- GC sample vials. Sample vials 1.5 ml, clearglass, art. no. 18081 Merck (Darmstadt, Germany). Vial, glass 12/BX (300 ml), part no. 5080-8779 Hewlett-Packard (Amsterdam, Holland).

5.2.4 Investigated cosmetics
Table 5.1 is a list of the ten products that were investigated. The Danish EPA is aware of names and companies from which the products originate.

Table 5.1
**Analysed products**

<table>
<thead>
<tr>
<th>MST Reg. Nr.</th>
<th>Product description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Hand lotion</td>
</tr>
<tr>
<td>A2</td>
<td>Facial lotion A</td>
</tr>
<tr>
<td>A3</td>
<td>Massage oil</td>
</tr>
<tr>
<td>A4</td>
<td>Facial lotion B</td>
</tr>
<tr>
<td>B1</td>
<td>Bergamot oil</td>
</tr>
<tr>
<td>B2</td>
<td>Jasmine oil</td>
</tr>
<tr>
<td>B3</td>
<td>Mint oil</td>
</tr>
<tr>
<td>B4</td>
<td>Mixed oil</td>
</tr>
<tr>
<td>C1</td>
<td>Hand and body lotion</td>
</tr>
<tr>
<td>C2</td>
<td>Scrub lotion</td>
</tr>
</tbody>
</table>

**5.3 Preparation of GC–MS equipment**

- Install a Rtx-20 capillary column (30 m × 0.25 mm i.d., DF = 0.25 mm liquid phase) in the GC–MS.
- Power-up the instrument as described in the instruction for DIAS (GC-MS No. 1) and heat the column up to a high temperature (300 °C) and stay there for approximately 3 hours.
- Flow (carrier gas, helium): 1.0 ml/min (10 psi);
- Injection volume: 1 µl;
- Split vent: 50 ml/min;
- Septum purge: 3.5 ml/min;
- Injection temperature: 250 °C;
Run time: 86 min;

Temperature program (see below).

An unmistakable name for the sample is filled out in the sample schedule on the Saturn GC–MS Workstation together with the processing method "Allergens 1" to be used for the analysis. Temperature program is given in the method "Allergens 1".

After equilibrating the column and the GC–MS has been programmed, a blind sample (100% \( \text{CH}_2\text{Cl}_2 \)) is injected and analysed with the programmed temperature program before starting analysis of product samples. The following temperature program is used for the GC-analysis on a Rtx-20 capillary column (DIAS No. 23):

Temperature program:
Initial temperature: 35 °C; Initial time:

<table>
<thead>
<tr>
<th>min rate (°C/min)</th>
<th>temperature (°C)</th>
<th>holding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>120</td>
<td>1.00</td>
</tr>
<tr>
<td>8.00</td>
<td>280</td>
<td>20.00</td>
</tr>
</tbody>
</table>

5.4 Preparation of GC equipment

Install a WCOT fused capillary column (50 m × 0.25 mm i.d., DF = 0.2 mm liquid phase: CP–Wax 52 CB) in the GC.

Supply \( \text{CH}_2\text{Cl}_2 \) in the Auto Injector for cleaning the injection needle.

Power-up the instrument as described in the instruction for DIAS (GC No. 3) and heat the column up to a high temperature (250 °C) and stay there for approximately 3 hours.

Flow (carrier gas, helium): 1.30 ml/min (21 psi);

Injection volume: 2 µl; Split vent: 54.1 ml/min;

Septum purge: 3.4 ml/min;

Injection temperature: 200 °C;

Detector temperature: 230 °C;

Run time: 117 min;

Temperature program (see below); Samples (vial numbers) to be analysed is programmed in the HP ChemStation.

The vial number of the sample and an unmistakable name for the sample is filled out in the sample schedule on the HP ChemStation together with the processing method "Allergens 2" to be used for the analysis. Temperature program is given in the method "Allergens 2".

After equilibrating the column and the GC has been programmed, a blind sample (100% \( \text{CH}_2\text{Cl}_2 \)) is injected and analysed with the programmed temperature program before starting analysis of product samples. The following temperature program is used for the GC-analysis
on a WCOT fused CP–Wax 52 CB capillary column (DIAS No. 26).

**Temperature program:**

<table>
<thead>
<tr>
<th>rate (°C/min)</th>
<th>temperature (°C)</th>
<th>holding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>80</td>
<td>0.00</td>
</tr>
<tr>
<td>2.00</td>
<td>220</td>
<td>15.00</td>
</tr>
</tbody>
</table>

5.5 Preparation of samples for GC and GC–MS analysis

5.5.1 Preparation of external standard solutions

Prepare minimum 10 ml of a solution of β-pinene (2), linalyl acetate (10), eugenol (16) and parthenolide (18) at a concentration of 1.0 ml/ml in double-distilled CH₂Cl₂. These solutions are stored in a freezer (−24 °C) until use, and will hereafter be named as the external standard solution 1, 2, 3 and 4, respectively (ESD-solution 1 and so forth). From the ESD-solutions, external standard curves are prepared, which are used for the quantification of compounds 1–18 in product samples. The selected compounds represent the different structural types of compounds investigated: β-pinene, the non-oxygenated monoterpenes 1–5; linalyl acetate, the oxygenated monoterpenes 8–13; eugenol, the phenols 14–17.

Furthermore, the ESD-solutions have been used to determine detection and quantification limits (see section 7.2), linearity (see section 7.3), accuracy (see section 7.4) and precision (repeatability) (see section 7.5) for the method DIAS ALLERGENS 1, date 21.12 2001.

5.5.2 Preparation of a standard solution containing potential contact allergens

A standard solution containing 1–18 was prepared for the control of the column and other parameters. 10 ml from each standard solution, with a concentration of 1.0 ml/ml in double-distilled CH₂Cl₂, were transferred to a GC vial.

5.5.3 Preparation of sample solutions of cosmetics containing plant extracts and/or essential oils

Creams: Approximately 500 mg of the solid cream is weighted out precisely in a 5 ml volumetric flask and 4 ml CH₂Cl₂ is added. The flask is then sealed with a glass stopper and the solution is thoroughly shaken for approx. 5 min and placed in the dark at 5° C. After 16 hours the solution is filtered and concentrated under a gentle flow of nitrogen to 300 ml which is transferred to a GC vial that is marked with a unmistakable characteristic for the sample.

Oils: 10 ml of the oil is transferred to a 1.5 ml volumetric flask and 1.0 ml CH₂Cl₂ is added. The flask is then sealed with a glass stopper and the solution is thoroughly shaken for approximately 1 min and 200 ml of this solution is transferred to a GC vial (300 ml) that is marked with a unmistakable characteristic for the sample.

5.6 Identification of potential allergens and/or irritants in cosmetics containing plant extracts and/or essential oils

Some of the compounds 1–18 (Figure 1) was identified in A3, B1, B2, B3, B4 and C1 by comparing their mass spectra and GC retention times with that of authentic standards (see Table).

However, in the remaining investigated commercial A1, A2, A4 and C2 neither of
the compounds 1–18 could be detected nor structurally related compounds (see Table 5).

6 Approval of the analysis
The analysis can be approved if it has been carried out as specified in this instruction and if no irregularities have occurred during the analysis and no error messages occur in the report.

6.1 Approval of the sample analysed
The sample analysed can be approved with respect to this analysis if:

- Compounds 1–18 (Figure 1) are fully separated and can be quantified.

- The profile of the chromatogram is not significantly different from the GC and GC–MS chromatograms given in Figure 2 and 3, respectively.

- Potential allergens and/or irritants in a sample are fully separated from other compounds and can be quantified. This can vary because very different samples are investigated by the method and consequently changes in the original temperature programs may be necessary.

Figure 2. GC chromatogram of a standard solution containing compounds 1–18. Compounds separated on a CP–Wax 52 CB capillary column. Parthenolide (18) does not elute on this column.
6.2 Approval, registration and filing the result
The result reports from the GC and GC–MS analysis incl. chromatograms, are handed over to the LM, who approves/rejects the analysis and the product tested. The LM states the results in the relevant certificates and files the documents and result reports in the appropriate binder. The technician performing the analysis states the final result in the laboratory journal as well.

7. Validity of the GC and GC–MS method
The GC and GC–MS method DIAS ALLERGENS 1, date 21.12 2001 has been validated for the following parameters: 1. robustness (column, flow and temperature), 2. detection and quantification limits, 3. linearity, 4. accuracy and 5. precision (repeatability).

7.1 Robustness
7.1.1 Column
Separation of the compounds in Figure 1 could be achieved on very different columns (Rtx-20 and CP–Wax 52 CB). See Figure 2 and Figure 3. The retention times of the individual compounds are, however, different due to differences in polarity of the columns. The sesquiterpene lactones, e.g. parthenolide, only elute on the Rtx-20 capillary column.

7.1.2 Flow
A lower flow (1.20 ml/min) and higher flow (1.40 ml/min), respectively, compared to normal flow of 1.30 ml/min did not significantly change the separation of the investigated compounds (Figure 1). These investigations were performed on a
WCOT fused capillary column (CP–Wax 52 CB).

### 7.1.3 Temperature
Small changes in the temperature program did not significantly change the separation of the investigated compounds (Figure 1).

Flow = 1.30 ml/min. Column: CP–Wax 52 CB. Temperature program.
Initial temperature: 32 °C; Initial time: 0.00 min

<table>
<thead>
<tr>
<th>rate (° C/min)</th>
<th>temperature (° C)</th>
<th>holding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.00</td>
<td>80</td>
<td>0.00</td>
</tr>
<tr>
<td>2.00</td>
<td>220</td>
<td>15.00</td>
</tr>
</tbody>
</table>

Flow = 1.0 ml/min. Column: Rtx-20.
Temperature program.
Initial temperature: 35 °C; Initial time: 2.50 min

<table>
<thead>
<tr>
<th>min rate (° C/min)</th>
<th>temperature (° C)</th>
<th>holding time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50</td>
<td>120</td>
<td>1.00</td>
</tr>
<tr>
<td>8.50</td>
<td>280</td>
<td>20.00</td>
</tr>
</tbody>
</table>

### 7.2 Detection and quantification limits
The determination of detection and quantification limits is based on visual evaluation. In order to determine the detection and quantification limits of compounds 1–18 (Figure 1), the ESD-solutions were diluted up to a factor of 6000. Based on these investigations the following detection and quantification limits were determined (see Table 1).

**Table 1. Detection and quantification limits for compounds 1–18.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Detection limits (µg/ml)</th>
<th>Quantification limits (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–7</td>
<td>~ 0.00010</td>
<td>~ 0.0002</td>
</tr>
<tr>
<td>8–13</td>
<td>~ 0.00010</td>
<td>~ 0.0002</td>
</tr>
</tbody>
</table>
7.3 Linearity

The samples for the determination of linearity were prepared from the respective ESD-solutions by diluting a specific volume of the ESD-solutions with CH$_2$Cl$_2$. Linearity was found for all ESD-solutions. For simplicity this is only shown for ESD-1 ($\beta$-pinene) and ESD-3 (eugenol), respectively (Figure 4 and Figure 5).

Table 2. Experimental parameters for determination of linearity using ESD-solution 1 ($\beta$-pinene).

<table>
<thead>
<tr>
<th>Concentration of $\beta$-pinene (mg/ml)</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1144</td>
<td>0</td>
</tr>
<tr>
<td>0.0572</td>
<td>2</td>
</tr>
<tr>
<td>0.0286</td>
<td>4</td>
</tr>
<tr>
<td>0.0143</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3. Experimental parameters for determination of linearity using ESD-solution 3 (eugenol).

<table>
<thead>
<tr>
<th>Concentration of eugenol (mg/ml)</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1421</td>
<td>0</td>
</tr>
<tr>
<td>0.0711</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 4. Linearity determination using ESD-solution 1 (β-pinene).

Figure 5. Linearity determination using ESD-solution 3 (eugenol).
7.4 Accuracy

Samples for the determination of accuracy were prepared from the ESD-solution 2 by addition of a known amount of the analyte (linalyl acetate) (see Table).

Table 4. Experimental parameters for determination of accuracy by addition of a known amount of linalyl acetate (LA) to a diluted ESD-solution 2.

<table>
<thead>
<tr>
<th>Added amount of LA (mg)</th>
<th>Amount of LA (mg) in the sample</th>
<th>Determination of LA (mg) in the sample</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.015</td>
<td>0.013</td>
<td>86.7</td>
</tr>
<tr>
<td>0.015</td>
<td>0.030</td>
<td>0.027</td>
<td>90.0</td>
</tr>
<tr>
<td>0.105</td>
<td>0.120</td>
<td>0.116</td>
<td>96.7</td>
</tr>
</tbody>
</table>

7.5 Precision (repeatability)

The repeatability was assessed at 2 concentration levels (4 replicates each) using the relaxing oil from B4. The 2 concentration levels are given by the total amount of potential allergens, which are:

concentration level 1 = 0.144 mg/ml;

concentration level 2 = 0.036 mg/ml.

Samples at the different concentration levels were prepared by diluting the stock solution (see section 5.5.2) of 144.0 mg/ml by a factor of 1000 and 4000, respectively, with CH$_2$Cl$_2$. 
Figure 6. Determination of the repeatability of the method at 2 concentration levels of the product B4. Mean of four replications ± standard dev. Relative standard deviation (RSD).

Concentration level 1: RSD 3.5%.
Concentration level 2: RSD 5.0%.

8. Concentration of contact allergens in cosmetics
The concentration of compounds 1–18 were determined in different cosmetics containing plant extracts and/or essential oils by using standard curves for the ESD-solutions 1–4, and the concentrations are shown in Table 8.1. The values are mean of two determinations with a variation less than 3%.

Table 8.1 Results from the quantification of the potential contact allergens and/or irritants (1–18) in cosmetics containing plant extracts and/or essential oils. Can be seen here.

9. References


