Standard Operation Procedure for the Determination of Release of Di-Isononylphthalate (DINP) in Saliva Simulant from Toys and Childcare Articles using a Head Over Heels Dynamic Agitation Device

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WARNING:

Phthalates are widely spread in the environment. They may be present in many solvents of analytical quality as well as in water. To avoid contamination the use of PVC tubing, pipeting balloons, rubber tubing etc. should be avoided. All plastic materials are to be suspected for the presence of interfering components, therefore the absence of potential interferences should be established before running analysis (using blank runs). Glassware should be thoroughly cleaned before use, preferably with a suitable, pure organic solvent.
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0 Introduction
Phthalates are commonly used as plasticisers in soft PVC articles such as toys and childcare articles, which may contain up to 50% of them. During use of the articles by babies, these plasticisers may leach into the saliva and consequently expose them to phthalates. Considering the low body weight and the age of the babies, the exposure to phthalates should not exceed a tolerable daily intake. Previous in-vivo studies on human volunteers (Dutch Consensus Group) using standard PVC disks containing di-isononylphthalate (DINP) as plasticiser have indicated the range of release that could be expected in-vivo for DINP on standard materials. Thus there is now a need to develop in-vitro mechanically based methodologies to test toys and childcare articles which will be able to simulate the range of releases observed in vivo.

The methods allow the identification and quantification of DINP in artificial saliva after mechanical agitation with plasticised PVC. A “head over heels” device was developed based on a rotational agitation principle originating in the Netherlands (Nutrition and Food Research Institute, TNO). The method was optimised by the European Commission Joint Research Centre (JRC) and validated with Member States’ laboratories. DINP was taken as model since it is currently the most commonly used phthalate in PVC toys and childcare articles.

1 Scope
This SOP describes a mechanical extraction procedure that is suitable for the determination of the release of plasticisers from baby toys using 2 analytical procedures to quantify the amount of phthalate released in
simulated saliva. The methods described in this SOP allow the identification and quantification of DINP in artificial saliva after mechanical agitation with plasticised PVC.

The mechanical procedure was calibrated against data obtained from in-vivo studies with plasticised PVC samples. The conditions of shaking were defined to obtain the mean value of the migration of DINP in the Dutch Consensus study in-vivo around 3μg/min/10cm². The above value had been in turn obtained by the summing of the mean release value and the standard deviation.

The analytical part of the method (extraction and analysis) used both HPLC and GC-MS. The GC-MS system allows for simultaneous identification of phthalate esters and their quantification as long as calibration curves have been established for the phthalates considered. An analysis on HPLC system was used to verify the quantification, since the system not capable at this point to discriminate between DINP and 2 other phthalate esters, namely di-isodecyl phthalate (DIDP) and di-ethylhexylphthalate (DEHP).

2 Principle

The test sample is mechanically treated with a saliva simulant solution made of an aqueous salt solution. After 30 min. of mechanical dynamic treatment have elapsed, the saliva extract is transferred to be extracted, while the flask and specimen are replenished with fresh saliva simulant, and subjected to another migration treatment. The pooled simulant solution extracts are subjected to two successive extractions using cyclohexane in a separatory funnel. The amount of DINP in the cylohexane solution is determined on GC-MS using a non-polar column, and by normal phase HPLC using UV detection at 225 nm. Quantification is achieved by using an external standard calibration procedure as well as an internal standard.

3 Reagents

WARNING: Phthalates are widely spread in the environment. They may be present in many solvents of analytical quality as well as in water. To avoid contamination the use of PVC tubing, pipeting balloons, rubber tubing etc. should be avoided. All plastic materials are to be suspected for the presence of interfering components, therefore the absence of potential interferences should be established before running analysis (using blank runs).

Glassware should be thoroughly cleaned before use, preferably with a suitable, pure organic solvent.

3.1 Analytes

3.1.1 Di-isononyl-phthalate (DINP)

Molecular weight: 418g/mol, Molecular formula: C_{26}H_{42}O_{4}

NOTE: DINP is a phthalate ester of isononyl alcohol, which is composed of a mixture of branched isomers and may contain in addition minor amounts of C_{7} up to C_{11} branched
alcohols. Thus it will appear as a complex mixture and may differ between different manufacturers.

3.1.2 Benzylbutyl phthalate, BBP (as internal standard)

3.2 Solvents

3.2.1 Iso-octane (HPLC grade)

3.2.2 Iso-propanol (analytical grade)

3.2.3 Cyclohexane (analytical grade)

3.2.4 Dioxane, HPLC grade (for flushing / cleaning of HPLC column)

3.2.5 Acetone (for pre-rinsing glassware)

3.2.6 Tetrahydrofuran (analytical grade)

3.2.7 Hexane (analytical grade)

3.3 Stock standard DINP solutions

3.3.1 Concentrated stock solution (ca. 5000 μg/ml)

Weigh to the nearest 0.1 mg approximately 100 mg of DINP (3.1.1) in a 20 ml volumetric flask and fill to the mark with cyclohexane (3.2.3). Mix carefully.

Calculate the actual concentration in mg DINP per ml solution.

Repeat the procedure to obtain a second standard stock solution.

3.3.2 Standard stock solution (ca. 500 μg/ml)

Transfer by means of a volumetric glass pipette 5 ml of the concentrated standard solution at 5000 μg/ml (3.3.1) into a 50 ml volumetric and fill to the mark with cyclohexane (3.2.3). Mix carefully and thoroughly.

Calculate the actual concentration in mg DINP per ml solution.

3.3.3 Dilute stock solution (ca. 50 μg/ml)

Transfer by means of a volumetric glass pipette 5 ml of the standard solution at 500 μg/ml (3.3.2) into a 50 ml volumetric and fill to the mark with cyclohexane (3.2.3). Mix carefully and thoroughly.

Calculate the actual concentration in mg DINP per ml solution.

3.4 Calibration samples of DINP for analysis (HPLC, GC-MS) in cyclohexane

Transfer by means of a volumetric glass pipette the volume of the dilute standard solution (3.3.3) into volumetric flasks as indicated in the table below and fill to the mark with cyclohexane (3.2.3). Mix carefully and
thoroughly. A high concentration of 50 ppm (directly from 3.3.3) may be used if it is proven to not bring interferences to the GC-MSD system by overloading.

Calculate the actual concentration in mg DINP per ml solution. The solutions thus obtained contain approximately the amount in μg of DINP per ml outlined in the “resulting concentration” column of the table.

Add a constant concentration of 1ppm of Internal standard solution of BBP (3.5, solution at 1000μg/ml), the volume to attain this constant concentration for the various dilutions is shown on the right column in the table below.

<table>
<thead>
<tr>
<th>ml of solution (μg/ml)</th>
<th>in flask vol (μl)</th>
<th>resulting sol (μg/ml)</th>
<th>μl of Internal Std (3.5) to add</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>50</td>
<td>20</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>20</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>20</td>
<td>10.0</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>20</td>
<td>5.0</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>20</td>
<td>2.5</td>
</tr>
</tbody>
</table>

NOTE: The above table is an example for preparing the solutions. By taking X ml (first column) of a solution of concentration Y (second column) into a flask of volume Z (third column) and filling to the mark with cyclohexane, one can obtain a resulting solution of V μg/ml (last column). The solutions may be prepared slightly differently, provided the amount of error on the glass pipette used is always minimised and all calibration concentrations come from one dilute standard to trace potential deviations. If the volume of the flasks for dilutions differ from above, the amount of internal standard should be adapted accordingly to remain at 1ppm final concentration in all dilutions.

Transfer approximately 1ml of the standard solutions into an HPLC vial and 1 ml of the standard solution into a GC vials (4.6).

Close the vials with a crimp cap with PTFE-liner.

Avoid any contact of the solution with the crimp cap.

3.5 Internal Standard solution of BBP (ca. 1000 μg/ml and 250μg/ml)

A solution of BBP (3.1.2) is prepared in cyclohexane at 1000μg/ml for spiking into the calibration solutions.

A one fourth dilution of the solution above is prepared to obtain a solution at 250μg/ml to be used as internal standard for spiking the saliva samples and blanks post-migration.

3.6 Standard solution of DINP in iso-propanol (ca. 1000 μg/ml) for recovery check

Solutions are prepared in iso-propanol (3.2.2) for spiking purposes in saliva.

Weigh to the nearest 0.1 mg approximately 50 mg of DINP (3.1.1) in a 50 ml volumetric flask and fill to the mark with iso-propanol (3.2.2). Mix carefully.
Calculate the actual concentration in mg DINP per ml solution.

3.7 Saliva simulant chemicals

3.7.1 Water deionised (Milli Q quality)

3.7.2 Calcium chloride, dihydrate, CaCl$_2$.2H$_2$O; $M_w = 147.02$ (e.g. Aldrich, 22,350-6*)

3.7.3 Magnesium chloride, hexahydrate, MgCl$_2$.6H$_2$O, $M_w$ 203.3 (e.g. Sigma M-9272*)

3.7.4 Potassium carbonate, $K_2$CO$_3$, $M_w$ 138.2 (e.g. Sigma P-4879*)

3.7.5 Potassium chloride, KCl, $M_w$ 74.55 (e.g. Sigma P-3911*)

3.7.6 Potassium phosphate, dibasic, trihydrate, $K_2$HPO$_4$.3H$_2$O, $M_w$ 228.2 (e.g. Sigma P-5504*)

3.7.7 Sodium chloride, NaCl, $M_w$ 58.44 (e.g. Sigma S-9888*)

3.7.8 Hydrochloric acid, $HCl$, dilute solution (e.g. 3M)

3.8 Simulated saliva salt solution

Prepare a solution in water (3.7.1) of the following composition:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>mmol/l</th>
<th>mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium chloride</td>
<td>MgCl$_2$</td>
<td>0.82</td>
<td>166.7</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl$_2$</td>
<td>1.0</td>
<td>147.0</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>$K_2$HPO$_4$</td>
<td>3.3</td>
<td>753.1</td>
</tr>
<tr>
<td>Potassium carbonate</td>
<td>$K_2$CO$_3$</td>
<td>3.8</td>
<td>525.2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>5.6</td>
<td>327.3</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>10.0</td>
<td>745.5</td>
</tr>
</tbody>
</table>

Weigh the required amount of salts taking into account the presence of water of crystallisation.

NOTE: The above table shows the amounts to be weighed for one litre taking into account the water of crystallisation.

Dissolve the potassium and sodium salts in ca. 900 ml distilled water (3.7.1), then add the calcium and magnesium salts.

Adjust the pH to 6.8 with diluted hydrochloric acid (3.7.8). Transfer to a 1 litre volumetric flask and fill to the mark with distilled water (3.7.1).

Store the solution in the dark.

NOTE: The solution should not be used for more than two weeks. After that time, a fresh solution should be prepared.

3.9 Sodium sulphate, anhydrous (as moisture trap)

* or equivalent product: the brand of the chemical is only indicated as an example.
4 Apparatus

NOTE: An item is listed only where it is special or made to a particular specification, the usual laboratory glassware and equipment being assumed to be available.

NOTE: Screw caps both new and re-used as well as crimp caps may contain interfering components. Contact of the lining of the crimp cap with the organic solution should be avoided at any time.

NOTE: Cleaning of metal implements: metal implements should be thoroughly cleaned, washed with acetone, and rinsed with iso octane before use and left to dry thoroughly. Examples of metal implements (not exhaustive): hollow punching press, stainless steel balls, tweezers, paper clips.

4.1 Metal stainless steel tweezers

4.2 Punching press

Capable of producing 23mm+/-0.2mm diameter disks from 1mm thick PVC sheeting

4.3 Conical glass separatory funnel of 250ml with glass stopper and Teflon faucet

NOTE: Separatory funnels using a glass faucet which requires the use of silicone and an O rubber ring should be avoided when possible, unless it can be proven there are no interferences.

4.4 General volumetric glassware (grade A) and glass stoppers (grade A)

4.5 Metal paper clip for weighing thin toy samples (e.g. bath books)

The weight of the metal clip should be approximately of 0.5g

4.6 Glass vials (2ml) suitable for chromatographic auto-sampler, and crimp caps with PTFE inlay

4.7 Micro injection syringe, 20 μl, 50μl and 250 μl (e.g. Hamilton)

4.8 Rotary evaporator or Kuderna Danish (KD) device

4.9 Pear or round bottom flask, 50 ml or less (for rotary evaporation) or dedicated glass containers (for KD device)

4.10 Volumetric flask of 50 ml

4.11 10 ml screw cap vials and caps with PTFE inlay
4.12 Analytical balance

4.13 Migration glassware

NOTE: Cleaning of glassware:
All glassware (laboratory glassware and migration glassware including both flask and caps) should be thoroughly cleaned, thoroughly rinsed several times with distilled water then rinsed with acetone and subsequently twice with 10 ml of cyclohexane. Then flask and cap should be dried for 1 h in a highly clean oven at 105 °C.

Laboratory bottles 250 ml with flat bottom, and a screw neck, provided with screw cap with PTFE lined rubber septum.

Dimensions:  
outside diameter: 70 mm  
total height of bottle: 138 mm  
height bottom to start of neck: 75 mm  
inside neck opening: 30 mm

Supplier: Schott Duran  
flask: Cat. nr.2180136  
screw cap: Cat. nr 2924028

NOTE: New glassware must be used since the effect of ageing and increased porosity are unknown and under investigation. Shape and dimensions of the flasks influence the mechanical impact of the test sample. Deviation from the prescribed type of flask may result in unreliable results. In the experiments, mild conditions should be used first, whereas stringent conditions should be kept for last.

NOTE: The flask and the inside of the screw cap must be free of interfering components. The flask and cap must be thoroughly cleaned following the protocol outlined in the "warning" section.

4.14 Dynamic migration device (Head over heel rotator)

Requirements:
- Must be capable of holding 250 ml bottles (4.13)
- Speed must be variable and able to maintain during the test period
- Radius from the centre of the rotating axis to the centre of the flask must be 150 mm

4.15 Analytical Determination

4.15.1 Gas chromatography with mass detector (GC-MS)

Preferably with an automatic on-column injector and a mass detector, or alternatively with a PTV or splitless injector. In any case however, the absence of interference or contamination must be established. A mass detector should be used capable of scan range 50 amu to 500 amu. The detector should be connected to an integrator.

The gas chromatographic capillary column must be capable of producing a symmetric peak of DINP and capable to separate DINP from peaks originating from sample matrix or extracting solvent.

NOTE: Depending on the type of equipment used for the determination, the appropriate operating conditions are to be established.

The following conditions and parameters are recommended:

Column: 30 m x 0.25mm I.D. x 0.25μm df HP-5MS.
Injection: cool on-column, splitless, 1 μl, oven track mode.
Oven: 50°C, 1 min., 30°C/min to 280°C, 15°C/min to 320°C, 3 min.
Carrier: helium, 1 ml/min (36.2 cm/s), constant flow.
Transfer-line: 325°C.
Detection: MS in SIM mode.

The following ions may be monitored, and DEHP DINP and DIDP should be monitored. Before starting a sequence of analyses, the instrument is checked by the injection of 1 μl cyclohexane (3.2.3). The analysis is performed using the same conditions as for sample analysis. The MS is operated in SIM mode. On the obtained chromatogram, ion extracted are m/e 149 for DBP, BBP and DEHP, m/e 293 for DINP and m/e 307 for DIDP. Record the peak areas and calculate the LOD (limit of detection) and LOQ (limit of quantification) for a signal to noise of (3:1).

<table>
<thead>
<tr>
<th>solute</th>
<th>DBP</th>
<th>BBP</th>
<th>DEHP</th>
<th>DINP</th>
<th>DIDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary ion</td>
<td>149</td>
<td>149</td>
<td>149</td>
<td>149</td>
<td>149</td>
</tr>
<tr>
<td>secondary ion</td>
<td>223</td>
<td>91</td>
<td>279</td>
<td>293</td>
<td>307</td>
</tr>
</tbody>
</table>

**NOTE:** Using an on-column injector, the column and mass detector are very sensitive to overloads. 50ppm has been shown to be able to overload the MSD lens and repeller. Thus it is recommended to lower the calibration curve to 10 or maximum 25 ppm maximum value in this case.

Using a splitless injector, it is advised to choose one of a volume as small as possible, preferably like a straight splitless liner of 2mm diameter 250 μl volume (e.g. for HP, part# 18740-80220) or even 1.5mm diameter 150 μl volume. Avoid the generic split/splitless liner of 4mm due to lack of sensitivity.

The following conditions have been found optimised in splitless using the 1.5mm 150μl volume splitless straight narrow “direct liner” (e.g. HP#18740-80200 or equivalent). In addition, the settings were the following (whenever possible on the brand of GC used):

| Mode:       | Pulsed splitless |
| Initial temperature: | 290°C |
| Pressure:   | 7.64 psi |
| Pulse pressure: | 35.0 psi |
| Pulse time: | 0.50 min. |
| Purge flow: | 20.0 ml/min |
| Purge time: | 2.0 min |
| Total flow: | 23.05 ml/min |
| Gas saver:  | on |
| Saver flow: | 20.0 ml/min |
| Saver time: | 2.5 min |

**4.15.2 High performance liquid chromatography (HPLC)**

Preferably with an automatic injector or injection loop (20 or 100 μl), and a variable UV detector, set to 225 nm, or a photodiode array detector scanning from 200-400 nm. The detector should be connected to an integrator.

The HPLC column must be capable of producing a symmetric peak of DINP and be able to separate DINP from peaks originating from sample matrix or extracting solvent.
NOTE: Depending on the type of equipment used for the determination, the appropriate operating conditions are to be established.

The following parameters are recommended:

Column: stainless steel 150 mm x 4.6 mm, filled with cyanopropyl coated spherical silica gel, particle size 5 μm., Hypersil BDS CPS (Shandon).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column temperature</td>
<td>30°C</td>
</tr>
<tr>
<td>Eluent</td>
<td>iso-octane (3.2.1)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1ml/min</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 μl</td>
</tr>
<tr>
<td>Detection</td>
<td>UV/diode array</td>
</tr>
<tr>
<td>Wavelength</td>
<td>200-400 nm, DINP measured at 225 nm</td>
</tr>
</tbody>
</table>

NOTE: Retention time obtained for DINP is approx. 4.5 min. Depending on the number of samples injected and the purity of the samples it may be necessary to clean the column periodically by washing with a mixture of iso-octane (3.2.1) and dioxane (9:1) to elute strongly retained components from the column. After reconditioning of the column with iso-octane (3.2.1) the samples analysis can be continued.

5 Procedures

5.1 Rapid identification of plasticisers in samples

Approximately 100 mg ± 5 mg of the sample is weighed accurately and dissolved in 10 ml of tetrahydrofuran (THF) (3.2.6), by vortexing 30 ± 5 seconds.

The polymer is precipitated with 20 ml hexane (3.2.7)

The solution is filtered through a 0.45μm disposable filter (e.g. nylon, polypropylene)

The portion of the clear THF/hexane solution is transferred into a capped vial and injected in GC-MS (see section 4.15.1)

A total ion current spectrum is recorded

The obtained GC-MS spectrum is compared to known spectra or phthalate ester standards to allow qualitative identification of phthalate ester plasticisers. The identity of the plasticisers is established based on retention time and reference phthalates.

For confirmation of the quantification of the plasticiser a 50 μl aliquot of THF/hexane solution is diluted in iso-octane and analysed by HPLC (4.15.2)

5.2 Sample preparation and conditioning

Five replicate test specimens should be analysed per material (toy) investigated, together with one blank and two recovery spikes. If it is not possible to take five test specimens from one test sample then more test samples should be taken to obtain five test specimens.
5.2.1 Cutting of test specimens

Select a flat as possible area of the article to be investigated and punch out five disks of approximately 10 cm² surface area using a 23 mm hollow punching press or cork borer.

NOTE: Use a Teflon surface to cut on, which allows for easy punching and “inert” surface.

Measure and record accurately the total surface area of each disk, taking both sides of the test specimen into account. If the thickness of the punched disk is greater than 1 mm then also the cutting edges should be taken into account.

A representative part should be taken from massive samples for analysis. The cutting edges should be as smooth as possible and loose particles should be removed beforehand.

Test specimens cut from thin materials may stick to the wall of the extraction container during rotation in the head over heals rotator. To prevent sticking, the test specimen should be supplied with some extra weight. For that purpose it is considered sufficient to pierce a little hole in the test specimen and to fix a metal paper clip (4.5) through the hole.

5.2.2 Conditioning of the test specimens

Store the disks in a glass container and allow the disks to recover from the mechanical treatment for at least 24 hours. The disks should not be stacked.

5.2.3 Preparation of test specimens

Rinse the cut disks by immersion for a few seconds in a beaker using tweezers with dionised water (3.7.1) and saliva simulant (3.7) in order to remove adhering particles.

Insert the test specimen disk in a flask (4.13) containing 50.0 ml of saliva simulant (3.8).

5.3 Dynamic migration testing

Blanks and recovery samples should be analysed daily or ideally with each series of samples, as described below.

NOTE: If, for any reason, test specimens are examined with deviating surface area, then the ratio of sample area to simulant should be maintained at 10 cm² to 50 ml. Although no analytical data are available it is considered that the total volume of simulant should not exceed 50 ml. Otherwise a larger size of extraction flask should be used.

WARNING: It is necessary to observe the samples during the agitation period because a test specimen may stick to the walls of the container.

5.3.1 Sample specimens

Place the 10 cm² disk (5.2) into the 50 ml saliva (3.7) at 20°C± 2°C using metal tweezers (4.1).

Close the flask and place the flask in a head over heels rotator (4.14).
Switch on the rotator, fixed at 60 ± 5 rpm, and allow to rotate for 30 ± 1 min.

After this period, immediately remove the disk from the flask (4.13) by means of a pair of tweezers (4.1).

Transfer the content of the flask into a closed separatory funnel (4.3)

Add fresh saliva simulant (3.7) to the flask (4.13)

Add the disk previously treated to the flask

Close the flask and place the flask in a head over heels rotator (4.14).

Switch on the rotator, fixed at 60 ± 5 rpm, and allow to rotate for 30 ± 1 min.

After this period, immediately remove the disk from the flask (4.13) by means of a pair of tweezers (4.1).

Add the content of the flask into the separatory funnel (4.3) already containing the first extract, and proceed to the extraction.

**NOTE:** Always place an even number of flasks in the head over heels rotator to avoid unbalanced rotation.

### 5.3.2 Blanks

Pipette 50 ml of fresh saliva simulant into a 250 ml flask or bottle (4.13). Treat the solution as described in the previous section, omitting the addition of a test specimen but following the second 30 minute replenishment extraction procedure.

Use the blank to correct the sample result if necessary.

### 5.3.3 Recovery samples

Transfer 50 ml of fresh saliva simulant (3.7) into a 250 ml flask or bottle (4.13)

Add by means of a 250 µl injection syringe (4.7) 150 µl of a standard solution of DINP at 1000 µg/ml in iso-propanol (3.6).

Assure that the tip of the syringe is submerged in the saliva simulant, and swirl before retracting the syringe from the saliva simulant. The saliva simulant thus obtained contains approximately 150 µg of DINP.

Close the flask and treat the mixture as described in the previous sections, omitting the addition of a test specimen but following the second 30 minute replenishment extraction procedure (no standard need be added again).

**NOTE:** The concentration of the spike DINP was made to be 15 µg/ml

### 5.4 Extraction from the saliva

**NOTE:** This SOP was designed for GC-MS and HPLC limits of quantification at 0.5 ppm for DINP and 0.01 ppm for BBP, and allowing quantification of releases from 1 to 10 µg/min even considering the integration of multiple peaks for DINP.
Add 20 ml of cyclohexane (3.2.3) to the bottle (4.13) used for migration which have been emptied of their simulant.

Close the bottle and shake vigourously to dissolve any absorbed phthalate. Remove the cap.

Add to the 250 ml separatory funnel (4.3) containing the 100 ml of saliva simulant.

Add by means of a 50 µl injection syringe (4.7) 40 µl of a standard solution of BBP in cyclohexane at 250 µg/ml (3.5) (except for blank sample)

Assure that the tip of the syringe is submerged in the solution, and swirl before retracting the syringe. The extract contains 10 µg of BBP in the saliva and solvent mixture.

Close the funnel and shake, vigorously, the stoppered 250 ml separatory funnel for approximately 2 ± 0.5 min., releasing pressure periodically (ca every 20 sec.).

Allow the phases to separate for 20 min.

Pour out the lower saliva simulant layer into a flask or bottle used for migration (4.13), by excess.

Pour the upper solvent layer into a 50 ml volumetric flask (4.10)

Transfer the saliva simulant from the migration glassware (4.13) back into the separatory funnel (4.5) for a second extraction.

Repeat the extraction procedure by adding another 20 ml of cyclohexane (3.2.3) to the bottle (4.13) used for migration which have been emptied of their simulant.

Swirl the solvent to absorb any phthalates on the sides of the glass vessel.

Add to the 250 ml separatory funnel (4.3) containing the 100 ml of saliva simulant.

Close the funnel and shake, vigorously, the stoppered 250 ml separatory funnel for approximately 2 ± 0.5 min., releasing pressure periodically (ca every 20 sec.).

Allow the phases to separate for 20 min.

Pour out the lower saliva simulant layer into a waste flask

Pour the upper solvent layer into the 50 ml volumetric flask (4.10) previously used for the first extraction. Fill up to the 50 ml mark with cyclohexane.

Add 1-2 g sodium sulphate (3.9) to the 50 ml volumetric flask (4.10).

**NOTE:** The sodium sulphate allows to trap potential moisture remaining from the extraction

Transfer a 20 ml aliquot of the cyclohexane extract to a pear round bottom flask or a KD flask (4.8).

**NOTE:** Care should be taken to avoid any transfer and contamination by the sodium sulphate
Transfer the flask (4.8) to a rotary evaporator or alternatively a Kuderna Danish (KD) apparatus and evaporate down to approximately 2 ml. Mild temperature conditions should be used (max. 40°C) using rotary evaporation.

NOTE: Care should be taken to avoid cross-contamination of the evaporating unit, therefore frequent solvent blanks should be run.

Remove from the concentrating unit and gently evaporate the remaining solvent to dryness using a flow of nitrogen at mild temperatures (<40°C) mild flow.

Add by means of a glass pipette 4.0 ml of cyclohexane (3.2.3), swirl thoroughly for approximately 30 seconds to entirely re-dissolve the phthalate ester including any which could be on the sides of the glass container.

Transfer 1ml of the solution into a vial for GC/MS and 1 ml into a vial for HPLC.

Close the vial with a crimp cap with PTFE-liner, ensuring caps are tightly crimped. Avoid any contact of the solution with the crimp cap.

Retain 8 ml of the remaining unconcentrated solution for potential re-analysis in screw-cap vials (4.11).

NOTE: The concentration of the internal standard was made to be 1µg/ml. If necessary the solution may require dilution to meet the limit of the calibration curve for unknown samples for DINP. The amount of internal standard must then be adjusted to remain constant at 1 µg/ml.

For blanks and recoveries, proceed as above without the disk on saliva alone for the blank and on fortified samples for recoveries. Determine and record recoveries.

5.5 Analytical determination

NOTE: When starting analyses, baseline stability and response linearity of the instrument should be examined.

NOTE: Each vial should be injected only once as it was found that interferences occurred upon a second injection from one and the same vial. If for any reason the analysis has to be repeated then a new vial should be filled and closed with a new crimp cap.

The same operating conditions of the GC-MS and HPLC system should be maintained throughout the analysis of all test samples and solutions.

5.5.1 Calibration samples

Prepare the standard solutions for calibration as described in section 3.4.

Transfer approximately 1ml of the standard solutions into a HPLC vial and 1 ml of the standard solution into a GC vials (4.6).

Close the vials with a crimp cap with PTFE-liner.

Avoid any contact of the solution with the crimp cap.

Inject each of the calibration samples (3.4) one into the GC-MS column (4.15.1), and its duplicate in the HPLC column (4.15.2).
Measure the peak heights or areas of DINP and of the Internal Standard in the chromatogram obtained.

**NOTE:** For GC-MS take m/z 149 for BBP and 293 for DINP

**NOTE:** For HPLC analysis, the use of the internal standard may be omitted as injection in HPLC is sufficiently repeatable.

Calculate the peak area ratio (PAR) for each of the calibration standards, by dividing the DINP peak area by the internal standard area.

Construct the calibration curve by plotting the peak area ratio against the concentration of DINP in the calibration samples in µg per ml cyclohexane.

Repeat with the second set of dilutions (duplicate set).

**NOTE:** The calibration curve should be rectilinear and the correlation coefficient should be 0.990 or better. If either of the two requirements is not met, fresh standard solutions should be prepared from the original stock solutions. Analysis of the solutions and construction of the calibration graph should be repeated.

**NOTE:** The calibration curves obtained in GC-MS tend to have a quadratic tendency, however, because of the concentration step placing the quantification in the middle of the curve, a linear regression should be used.

**NOTE:** Peak height was found to give better calibration curves for HPLC. Peak Areas should be used for GC-MS analyses.

**NOTE:** Calibration solutions should be injected frequently during the analysis of saliva simulant samples. At least 1 calibration solution should be injected on every ten saliva simulant samples.

5.5.2 Test samples, blank and recovery solutions

Extracts of the saliva simulant samples, blanks and recoveries are analysed.

Place the vials with the extraction solutions run the analysis using the conditions set out in section 4.15.1 (GC-MS) and 4.15.2 (HPLC).

Measure the peak areas (GC-MS) or peak heights (HPLC) of the DINP peak and Internal Standard peaks respectively in the chromatograms obtained.

Calculate the ratio of the peaks (GC-MS) by dividing the DINP peak area by the internal standard area.

Use the measured peak ratio –GC-MS (height, HPLC) as obtained above in the following formula.

If the regression line equation is

\[ y (\text{PAR}) = a \times x [\mu g/ml] + b \]

then the DINP concentration in cyclohexane (µg/ml) is

\[ C_{\text{DINP, solvent}} = \frac{(y-b)}{a} \]

5.5.3 Calculation of the DINP release from the test specimen

The release of DINP should be expressed in µg/min taking into account 10 cm² of surface area of the test specimen.
Calculate the release as follows:

\[
\text{Release [\mu g/min]} = \frac{C_{\text{DINP, solvent}} [\mu g/ml] \times V_{\text{extract}} [ml] \times 10 [cm^2]}{t [min] \times A [cm^2]} \times F
\]

In which:

\(C_{\text{DINP, solvent}}\): concentration of DINP in cyclohexane  
\(V_{\text{extract}}\): volume of extraction (ml): 50ml.  
\(T\): time of experiment (60 minutes)  
\(A\): area of test specimen (cm²)  
\(F\): Factor for concentration step F= 0.2

5.5.4 Calculation of recoveries

\[
\text{Recovery (\%)} = \frac{C_{\text{DINP, solvent}} [\mu g/ml] \times V_{\text{extract}} [ml] \times 100}{\text{Concentration of recovery solution [\mu g/ml]} \times 1 [ml]} \times F
\]

6 Reporting

The report should contain the reference to this method, and all information necessary for the complete identification of each sample, including the code number, description of the sample, area taken and weight. It should also contain the sample arrival date or sampling date, the method of sampling, the date of analysis together with note on any intervening storage conditions.

The results from GC-MS analysis on the sample, reference material and recovery solution should be transposed to a spreadsheet for calculating and reporting.

The results should then be reported in \(\mu g/min/10cm^2\), reporting all individual results as well as the mean of the five determination satisfying the repeatability criterion in section 7.

The report should mention any deviations from the method description and reasons for variations, and relevant comment on the test results.

Should results be unexpected and subject to doubt the analyses should be performed using a standard reference disks which can then be used as a calibrant to the performance of the method and/or operator.

The report should also identify the laboratory conducting the test and the name of the analyst.

The responsibilities of the officer in charge are to explain the work and tests to be performed, and supply a written experimental design showing the chronology and requirements during the experiments. The officer is also responsible for performing a visual observation during one experiment in order to observe the proper execution of the tests. The officer is then responsible for providing the electronic data sheet to input.
the results, and to interpret, perform statistical analysis when necessary and write the report.

The responsibilities of the technician(s) responsible for the analysis are to execute the experiments, check the functioning of instruments and availability of consumables, state of materials needed, receive and store the samples and standards, check the chromatograms obtained and input the data into the proformat provided.

7 Repeatability and reproducibility

The method was validated at the European Level on 14 laboratories using both PVC reference material and 5 toys. The maximum repeatability relative standard deviation (RSDr) was found to be 10%. Since the determination cannot be done on several real toy samples, the repeatability is expressed for the 5 replicates from one sample within laboratories (value for all participating labs, according to the definition of ISO 5725).

In addition standard PVC reference samples (n= 10) of known homogeneity were analysed in 2 various days, and the maximum RSDr was found to be 8%.

The reproducibility relative deviation (RSDr) was found to be 30% using a reference PVC material containing 38% of DINP.

8 Safety

General safety instructions should be followed at all times; if in doubt, advice must be sought from the officer in charge. The following specific hazards must be taken into consideration: use of sharp blades, use of solvents

All appropriate protective safety equipment should be worn and a fume cupboard must be used.

9 References


10.1 Annex 1 – Example of a prototype head over heels apparatus

10.2 Annex 2 – typical HPLC chromatogram showing the peak of DINP and that of the internal standard
10.3  Annex 2 – typical GC-MS chromatogram showing the extraction of ion 293, 307 and 149.
Mission of the JRC

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.