JRC Technical Notes

Determination of bis-(2-ethylhexyl) phthalate (DEHP) in sports drinks by isotope dilution headspace solid phase micro extraction gas chromatography mass spectrometry (HS-SPME-GC-MS)

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1 SCOPE AND APPLICATION

The method is suitable to determine bis-(2-ethylhexyl) phthalate (DEHP) in sports drinks at concentrations between 3 mg/L and 100 mg/L.

Note: The urgency of the request for method development did not allow to optimise method parameters with regard to e.g. run time.

2 SAFETY

Bis-(2-ethylhexyl) phthalate is harmful to humans

Protective equipment as laboratory coat, and safety glasses have to be used.

All handlings of DEHP and organic solvents should be performed in a fume hood with adequate air flow.

3 PRINCIPLE

A test portion is diluted in 10 mL screw cap vials with 5.0 mL methanol of proven quality, and isotopic labelled DEHP is added. A portion of the diluted sample is transferred into 22 ml headspace vials suitable for headspace solid phase micro extraction. The sample is then equilibrated and extracted by solid phase micro extraction. Measurement of the analyte is performed by gas chromatography mass spectrometry in single ion monitoring mode.

Note: DEHP is an ubiquitous substance. It is contained in at least small amounts in solvents, water, air, and many kinds of laboratory consumable, especially when made of plastic. Recommendations on how to deal with blank problems are specified in the course of this document.

4 APARATUS

4.1 HS-SPME-GC-MS SYSTEM

4.1.1 HS-SPME AUTOSAMPLER :

X-Y-Z robot capable of performing solid phase micro extraction, equipped with a 24 position sample tray and 6 position compartment for sample agitation and thermostatisation; SPME fibre holder; SPME fibre: PDMS 100 µm.

4.1.2 INJECTION PORT

PTV injection port with septum less head; SPME liner.

4.1.3 GC COLUMN

DB 5 MS, 30 m x 0.25 mm internal diameter and 0.25 µm film thickness, or equivalent.

4.1.4 MASS SPECTROMETER

Single quadrupole mass spectrometer operating in electron ionisation mode at 70 eV and capable of performing single ion monitoring (SIM).

4.1.5 DATA ACQUISITION AND ANALYSIS SYSTEM

Suitable data collection and evaluation software.

4.2 CALIBRATED MICROBALANCE

with a readability of 0.001 mg

4.3 CALIBRATED ANALYTICAL BALANCE

with a readability of 0.01 mg.

4.4 CALIBRATED POSITIVE DISPLACEMENT PIPETTES

Capacity 1000 µL and 5000 µL

Note: The pipette tips applied during the development of this study did not release significant amounts of DEHP. However the release of DEHP has to be checked prior to their application.

4.5 GLASS MICROLITER SYRINGES WITH GLASS OR METAL PLUNGERS

Volumes of 25 µL, 50 µL, 100 µL and 500 µL to 1000 µL

4.6 GLASSWARE:

Volumetric flasks with glass stoppers, volume 10 mL, 25 mL, 50 mL, 100 mL etc., according to ISO 1042.

Single use glass headspace vials with a volume of 22 mL, with appropriate PTFE lined septum and aluminium crimp cap, compatible with the HS-SPME sampler (4.1.1).

Single use screw cap vials of 10 mL with srew caps and PTFE lined septum

Glass beakers of different size

Large desiccators for the storage of cleaned glassware

With the exemption of headspace vials, which are applied as supplied, all reusable glassware is firstly cleaned in a laboratory dishwasher and then thoroughly rinsed with methanol and *n*-hexane. The 10 mL screw cap vials are rinsed with *n*-hexane prior to their use. All glass ware is stored in a desiccator over aluminium oxide (5.5). Glassware for standard preparation and sample extraction is rinsed twice with a small amount of, depending on the use, either methanol or *n*-hexane.

5 REAGENTS AND STANDARDS

Chemicals should be at least of pro analysis grade. Chemicals used for chromatography should be of LC-MS grade. All chemicals have to be checked prior to their application for contamination with DEHP.

5.1 DEUTERIUM-LABELLED BIS-(2-ETHYLHEXYL) PHTHALATE

Deuterium content >98% , supplied in screw cap vials with 10 mg analyte content

5.2 NEAT BIS-(2-ETHYLHEXYL) PHTHALATE

purity >99.5%, supplied in screw cap vials with 1000 mg analyte content

5.3 METHANOL

HPLC grade for extraction, LC-MS grade for chromatographic use

5.4 *N*-HEXANE

pro analysis grade, stored over 20 g/L aluminium oxide (10 s shaking prior to use).

5.5 ALUMINIUM OXIDE

The aluminium oxide is conditioned at 300 °C for 24 hours prior to use, and stored in a desiccator.

6 STANDARDS

Note: The standard concentrations given below are just indicative. The broad variability of the DEHP-d4 content supplied per vial makes the realisation of the indicated stock solution concentrations difficult. Therefore both the volumes of the standard preparation scheme and the concentration levels may be adopted to the actual situation.

Note: All standard solutions are stored refrigerated. Allow standard solutions to get to ambiant temperature before further application.

6.1 STOCK STANDARD SOLUTION OF DEHP IN METHANOL (1000 µG/ML)

Weigh to the nearest of 0.001 mg about 50 mg DEHP (5.2) into a 50 mL volumetric flask, and make up to volume with methanol. This solution can be stored at below 10 °C for at least 3 months.

6.2 INTERMEDIATE STANDARD SOLUTION OF DEHP IN METHANOL (150 µG/ML)

Transfer 1500 µL of the stock solution of DEHP in methanol (6.1) (1000 µg/mL) to a 10 mL volumetric flask and make up to volume with methanol.

6.3 STOCK STANDARD SOLUTION OF DEHP-D4 IN METHANOL (1000 µG/ML)

Record the tare weight of a 10 mL volumetric flask (incl. glass stopper) on an analytical balance. Rinse the flask twice with methanol, and add finally about 2 mL of methanol. Record the actual weight including the methanol.

Remove the label from the DEHP-d4 vial and clean the surface with acetone. Record the weight of the vial with the DEHP-d4 on a microbalance. It is advisable to perform repeated weighing of the vial. Open the vial and add with a microliter syringe methanol to the viscous liquid. Transfer with the microliter syringe the methanol from the DEHP-d4 vial into the 10 mL volumetric flask. Rinse the DEHP-d4 vial incl. screw cap with methanol by closing the vial and shaking it for a few seconds. Repeat the rinsing step another two times. Transfer all rinsing solutions into the volumetric flask, and fill it up to the mark with methanol. Record the total weight of the flask incl. glass stopper. Record the tare weight of the DEHP-d4 vial on the microbalance after evaporation of residual rinsing solvent. Weight constancy was usually found after leaving the vial open for several hours. Repeated weighing is advisable.

6.4 Calibration standards

6.4.1 Calibration standard stock solutions

Note: The given concentration levels are only indicative. The real concentrations have to be calculated from the actual concentrations of the stock standard solutions and intermediate standard solution.

Standard 0 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 3 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 500 µL of intermediate standard solution of DEHP in methanol (6.2; 150 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 9 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 1500 µL of intermediate standard solution of DEHP in methanol (6.2; 150 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 20 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 500 µL of stock standard solution of DEHP in methanol (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 40 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 1000 µL of stock standard solution of DEHP in methanol (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 60 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 1500 µL of stock standard solution of DEHP in methanol (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 80 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 2000 µL of stock standard solution of DEHP in methanol (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

Standard 100 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in methanol (6.3; 1000 µg/mL) and 2500 µL of stock standard solution of DEHP in methanol (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with methanol.

6.4.2 Dilution of calibration standard stock solutions

The concentration of the above calibration standard stock solutions is too high to be used directly for HS-SPME-GC-MS analysis. Therefore all calibration standards are diluted prior to an analysis sequence with methanol from the same batch that is used to dilute the test samples:

Pipette for each calibration standard with a calibrated positive displacement pipette 5000 µL methanol into a 10 mL srew cap vial and add 1000 µL of the calibration standard solution ("Standard 0 µg/mL" to "Standard 100 µg/mL"). Close the vial with the screw cap and shake gently.

Note: To avoid contact of the standard solution with the septum, the glass can be covered with aluminium foil prior to closing with the screw cap.

Pipette with microliter syringe or calibrated positive displacement pipette (100 µL tip) 20 µL of the diluted calibration standard solution into 22 mL head space vials and close the vial with an aluminium crimp cap with PTFE lined septum.

7 PROCEDURE

7.1 TEST SAMPLE PREPARATION

Pipette with a calibrated pipette (4.4) 1000 µL of sports drink sample into a 10 mL glass vial with screw cap and PTFE lined septum. Add with a suitable pipette 5000 µL of methanol (5.3) and with a microliter syringe (4.5) 20 µL of the DEHP-d4 stock standard solution in methanol (**Error! Reference source not found.**).

Homogenise the mixture. Prevent contact of sample with the PTFE lined septum by covering the vial's opening with aluminium foil prior to closing. Transfer consequently a 20 µL aliquot of the solution into a suitable HS-SPME autosampler vial and analyse by HS-SPME-GC-MS in selected ion monitoring mode.

Test samples shall be prepared in duplicate.

Note: The added methanol served as dilution agent, and provided better extraction efficiencies, which is attributed to the breakup of micelles.

7.2 PROCEDURAL BLANK SAMPLE

The procedural blank sample consist of 5000 µL of methanol (5.3) spiked with 20 µL of stock standard solution of DEHP-d4 in methanol (**Error! Reference source not found.**). A calibrated positive displacement pipette (4.4) is used for sampling of the methanol and a microliter syringe (4.5) for the transfer of the stock standard solution of DEHP-d4 in methanol (**Error! Reference source not found.**). This sample is then transferred into 22 mL HS-SPME autosampler vials as described before (see 7.1) and analysed by HS-SPME-GC-MS. The DEHP content of the procedural blank sample is subtracted from the DEHP content of the test samples. However it shall not exceed 30 % of the DEHP content of "Standard 3". If this is not the case then root-cause-analysis has to be performed and the source of contamination has to be eliminated.

Procedural blank samples shall be prepared for the proper establishment of background contamination at least in triplicate

Note: Methanol for the preparation of the procedural blank sample should stem from the same batch as the methanol used to dilute samples and to prepare standard solutions.

7.3 HS-SPME-GC-MS DETERMINATION

7.3.1 GC-MS CONDITIONS

For a successful analysis it is of paramount importance that the instrument is in good conditions and that all instrumental parameters are optimised.

The following GC parameters were successfully applied for the determination of DEHP in sports drinks. Representative chromatograms are presented in Annex 1.

HS-SPME parameters	.
Fibre type	100 µm PDMS
Incubator	Agitator
Extraction Temperature	90 'C
Extraction Time	10 min
Agitator On Time	60 s
Agitator Off Time	15 s
Agitator Speed	250 rpm
Vial penetration depth	21 mm
Injection penetration	43 mm
Desorption time	180 s (~100 mL/min split flow after 60 s)
Fibre bake out	Needle heater
Fibre bake out temp	250 °C
Fibre post run bake out time	4 min
Fibre type	100 µm PDMS
Incubator	Agitator
Extraction Temperature	90 °C
GC parameters	
GC Column	DB 5 MS, 30 m x 0.25 mm i.d., 0.25 µm d.f.
Carrier gas	He, 1.0 mL/min constant flow
Temperature programme	65.0 °C/1.0 min - 20.0 °C/min - 300.0 °C/2.0 min
GC-inlet	PTV with septum less head
Inlet temperature	275 °C, constant
Desorption time	3 min
Injection mode	Splitless for 1.0 min, afterwards split with ratio 1 : 100
Total run time	14.5 min
MS parameters Error! Bookmark not defined.	
Mass filter	Quadrupole
Ionisation	Electron Ionisation, 70 eV
Operation mode	Selected ion monitoring (SIM)
Solvent delay	7 min
Recorded ions (dwell time)	m/z= 149 (0.08 s), 153 (0.08 s), 167 (0.10 s), 279 (0.10 s)
GC-Interface temperature	300 °C
Ion source temperature	280 °C
Quadrupole temperature	150 °C

7.3.2 Analysis sequence:

Inject at the beginning of each sequence at least twice methanol (5.3), in order to clean the system. Inject then the calibration standards "Standard 0", followed by "Standard 3" for the evaluation of the system suitability. Afterwards the test samples, procedural blank samples, quality control samples, and calibration standards are injected in random order. Inject after maximum 10 samples methanol (5.3) to identify potential carry over.

7.3.3 System suitability

The complete elimination of DEHP background is difficult to achieve. It is however of minor importance with regard to the relative proportion of background levels and analyte concentrations in the samples and extracts, which exceed usual background levels by far. However phthalates might be released also from some GC parts or might enter the GC via the carrier gas supply.

Therefore the GC-MS system should be inspected at the beginning of each analysis sequence for its suitability to analyse the test samples. The system is considered suitable when the DEHP peak abundance of "Standard 0" does not exceed 20

% of the DEHP peak abundance of "Standard 3". If this is not the case then root-cause-analysis has to be performed and the source of contamination has to be eliminated.

8 IDENTIFICATION AND CALCULATION OF RESULTS

The peak identity is confirmed by comparison of the peak ratios for m/z 149, and 167 from sample extracts and standard solutions. The ratios should not differ more than ± 20% from those obtained for standard solutions.

Calibration by the internal standardisation is applied for the determination of DEHP. A calibration graph is constructed in which the ratio of the areas of DEHP and DEHP-d4 is plotted against the ratio of the concentrations of DEHP and DEHP-d4 in the respective calibration solution. The calibration function is determined by linear regression.

$$\frac{A_{DEHP}}{A_{DEHP-d4}} = a * \frac{C_{DEHP}}{C_{DEHP-d4}} + b \qquad \text{Equation 1}$$

where

A_{DEHP} is the area of the native DEHP peak (of m/z 149)

$A_{DEHP\text{-}d4}$ is the area of the DEHP-d4 peak (of m/z 153)

a is the slope of the calibration function

C_{DEHP} is the concentration of native DEHP

$C_{DEHP\text{-}d4}$ is the concentration of labelled analogue DEHP-d4

b is the intercept of the calibration function

Calculate for each sample the amount of DEHP that was extracted from the sample (X_{DEHP}) using the following equation:

$$X_{DEHP} = \frac{\left(\frac{A_{DEHP}}{A_{DEHP-d4}} - b\right)}{a} * X_{DEHP-d4} \qquad \text{Equation 2}$$

where

X_{DEHP} is the concentration of DEHP (in µg/mL ~ mg/L) of the sample.

A_{DEHP} is the area of the native DEHP peak corresponding to peak of m/z 149 of the test sample

$A_{DEHP\text{-}d4}$ is the area of the DEHP-d4 peak corresponding to peak of of m/z 153 of the test sample

$X_{DEHP\text{-}d4}$ is the absolute amount (in µg) of internal standard (DEHP-d4) added to the test sample

a is the slope of the calibration function

b is the intercept of the calibration function

Calculate according to equation 2 the DEHP content in the procedural blank samples and subtract the average content of the procedural blank samples from the results of the test samples.

The results of the test samples are reported corrected for the background contamination to three significant figures. The reporting unit is mg/L

9 QUALITY CONTROL

For each batch of samples the following controls are used:

9.1 LABORATORY REFERENCE MATERIALS

Proper spiked sports drink matrices or control samples are recommended for use as laboratory internal reference materials. The DEHP content of the laboratory reference material should be between 30 mg/L and 50 mg/L.

For this reason add 2000 µL of stock standard solution of DEHP in methanol (**Error! Reference source not found.**) into a 50 mL volumetric flask and make up to volume with a blank or at least only low contaminated sports drink.

9.2 CONTROL CHART.

The results for the laboratory reference materials should be monitored in control charts. Acceptable results should be within the limits of 3 times the intermediate precision standard deviation of the method.

10 METHOD PERFORMANCE

The following data shall be generated under the conditions described in this method:

10.1 LINEARITY

The calibration function was linear over the whole working range. The correlation coefficients of the different calibrations was greater than 0.995. Matrix effects were not found. Therefore instrument calibration can be performed with standards in methanol.

10.2 REPEATABILITY AND INTERMEDIATE PRECISION

Repeatability was estimated from six replicate analyses each of a sports drink sample spiked with DEHP to about 5 mg/L, 50 mg/L and 100 mg/L. The analyses were carried out on a single day by one operator.

The repeatability relative standard deviation were at

DEHP concentration level 5 mg/L: 3.6 %

DEHP concentration level 50 mg/L: 2.4 %

DEHP concentration level 100 mg/L: 2.7 %

Intermediate precision was estimated from analysis on two different days (with one week in between) of six replicates of sports drink samples spiked with DEHP to about 5 mg/L, 50 mg/L and 100 mg/L. The analyses were carried out on the different days by applying different positive displacement pipettes and different standard solutions of DEHP-d4 in methanol. Also calibration solutions were freshly prepared.

The intermediate precision relative standard deviation were at

DEHP concentration level 5 mg/L: 6.0 %

DEHP concentration level 50 mg/L: 4.7 %

DEHP concentration level 100 mg/L: 3.2 %

10.3 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The limit of detection and limit of quantification are far below the working range. Therefore only the S/N ratios of the DEHP peaks (m/z=149) at the lowest level of the calibration curve is given here.

The root mean square signal-to-noise ratio (RMS S/N) was 8263.

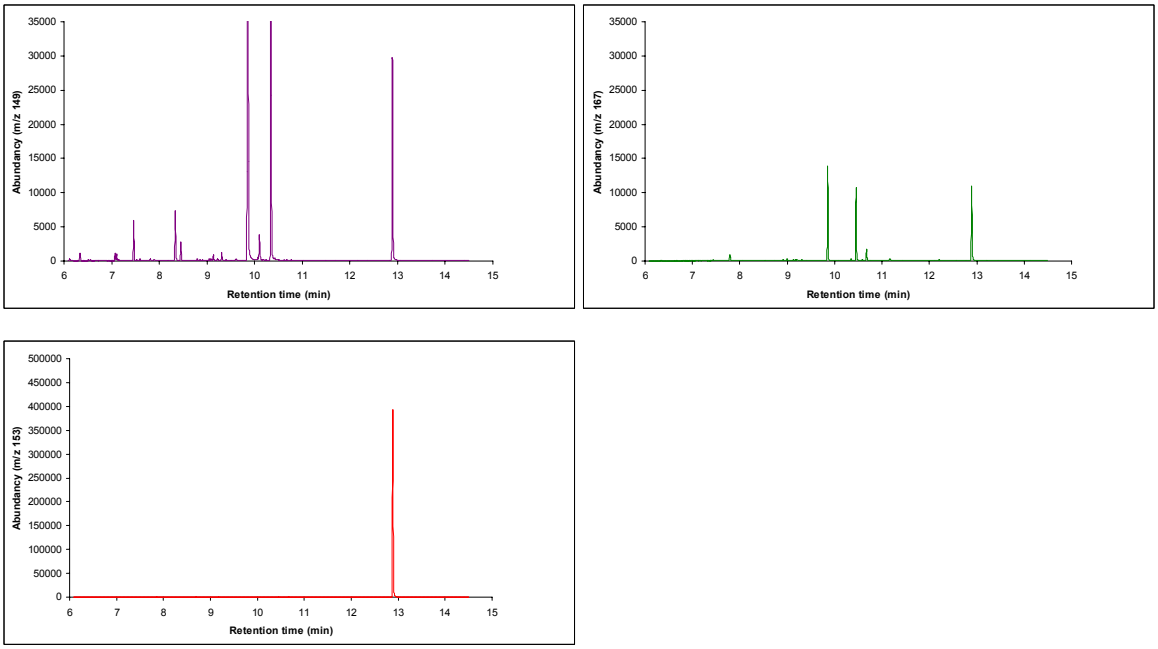
10.4 RECOVERY

The apparent relative recoveries of the determinations of the DEHP contents of the spiked samples, corrected for the procedural blank results, were in the range of 104 ± 6% .

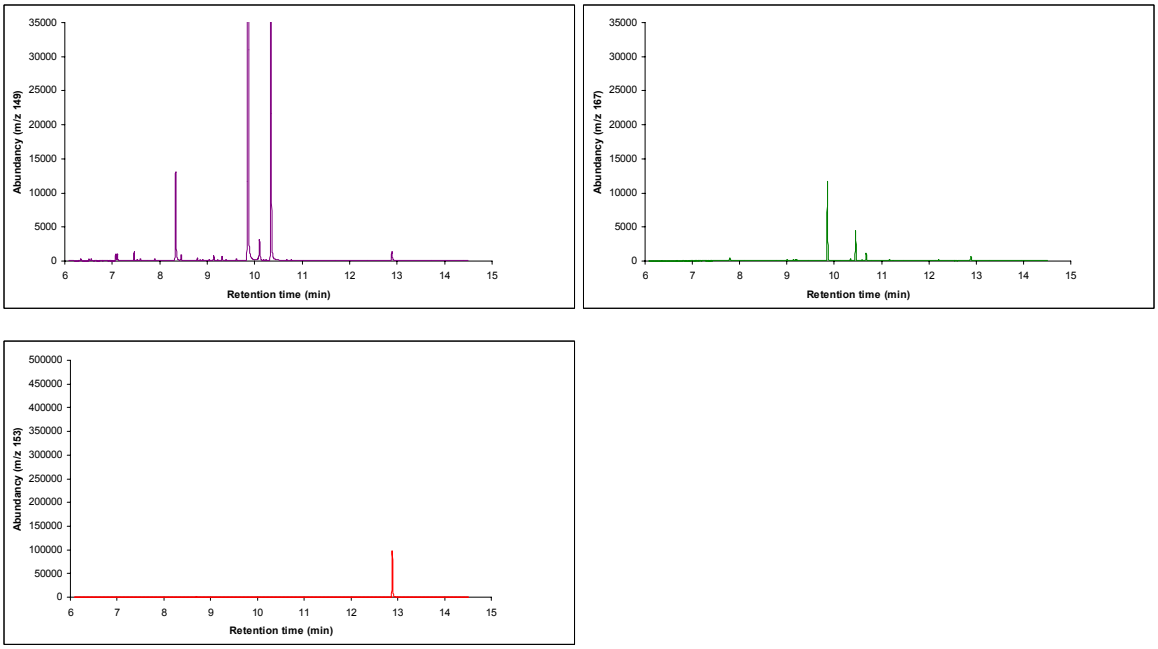
ANNEX 1

Example of HS-SPME-GC-MS chromatogram of the DEHP content analysis of sport drinks, a) spiked at 5 mg/L; b) blank sample

a)



b)



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