JRC Technical Notes

Determination of bis-(2-ethylhexyl) phthalate (DEHP) in sport drinks by isotope dilution ultra high performance liquid chromatography atmospheric pressure chemical ionisation tandem mass spectrometry (UHPLC-APCI-MS/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 8 mL screw cap vials with methanol of proven quality, and isotopic labelled DEHP is added. The sample is then extracted with *n*-hexane by vigorously shaking. Measurement of the analyte is performed by isotope dilution ultra high performance liquid chromatography atmospheric pressure chemical ionisation tandem mass spectrometry in selected reaction 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 UHPLC-MS/MS SYSTEM

4.1.1 AUTOSAMPLER :

Capable of injecting 2 µL of sample.

4.1.2 UHPLC SYSTEM

Chromatographer with UHPLC performance characteristics.

4.1.3 UHPLC COLUMN

Waters Acquity UPLC BEH phenyl , 150 mm x 1,0 mm internal diameter and 1,7 µm particle size, or equivalent.

4.1.4 MASS SPECTROMETER

Triple quadrupole mass spectrometer operating in positive atmospheric pressure chemical ionisation mode (APCI+) and capable of performing selected reaction monitoring (SRM).

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 WRIST ARM SHAKER

8 position, with adjustable shaking frequency and timer

4.7 GLASSWARE:

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

Single use glass screw cap vials of 8 mL with screw caps equipped with PTFE lined septa,

Single use 2 mL autosampler vials

Glass beakers of different size

Large desiccators for the storage of cleaned glass ware

With the exemption of autosampler 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 8 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.

5.6 WATER

Water for use in chromatography should be of LC-MS grade.

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 *N*-HEXANE (1000 µG/ML)

Weigh to the nearest of 0.001 mg about 50 mg DEHP (**Error! Reference source not found.**) into a 50 mL volumetric flask, and make up to volume with *n*-hexane. This solution can be stored at below 10 °C for at least 3 months.

6.2 INTERMEDIATE STANDARD SOLUTION OF DEHP IN *N*-HEXANE (150 µG/ML)

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

6.3 Stock standard solution of DEHP-d4 in *n*-hexane (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 *n*-hexane, and add finally about 2 mL of *n*-hexane. Record the actual weight including the *n*-hexane.

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 *n*-hexane to the viscous liquid. Transfer with the microliter syringe the *n*-hexane from the DEHP-d4 vial into the 10 mL volumetric flask. Rinse the DEHP-d4 vial incl. screw cap with *n*-hexane 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 *n*-hexane. 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 Stock standard solution of DEHP in methanol (1000 µg/mL)

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

6.5 Stock standard solution of DEHP-d4 in methanol (1000 µg/mL)

Repeat the standard preparation as detailed above for the DEHP-d4 standard in *n*-hexane (in 6.3) with methanol as solvent.

6.6 Calibration standards

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 *n*-hexane (0; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane.

Standard 3 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 500 µL of intermediate standard solution of DEHP in *n*-hexane (6.2; 150 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane.

Standard 9 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 1500 µL of intermediate standard solution of DEHP in *n*-hexane (6.2; 150 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane.

Standard 20 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 500 µL of stock standard solution of DEHP in *n*-hexane (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane..

Standard 40 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 1000 µL of stock standard solution of DEHP in *n*-hexane (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane.

Standard 60 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 1500 µL of stock standard solution of DEHP in *n*-hexane (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane

Standard 80 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 2000 µL of stock standard solution of DEHP in *n*-hexane (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane

Standard 100 µg/mL: Add with an appropriate syringe or pipette 500 µL of stock standard solution of DEHP-d4 in *n*-hexane (0; 1000 µg/mL) and 2500 µL of stock standard solution of DEHP in *n*-hexane (6.1; 1000 µg/mL) to a 25 mL volumetric flask and make up to volume with *n*-hexane

7 PROCEDURE

7.1 TEST SAMPLE PREPARATION

Pipette with a calibrated pipette (4.4) 1000 µL of sports drink sample into a 8 mL glass vial with screw cap and PTFE lined septum. Add with a suitable pipette 2000 µL of methanol (5.3) and with a microliter syringe (4.5) 50 µL of the DEHP-d4 stock standard solution in methanol (6.5).

Homogenise the mixture and add afterwards with a suitable calibrated pipette 2000 µL of *n*-hexane to the sample. Prevent contact of *n*-hexane with the PTFE lined septum by covering the vial's opening with aluminium foil prior to closing it with the screw cap. Shake the sample firstly shortly by hand and then for 1 hour on a wrist arm shaker (4.6) at highest shaking frequency. Other instruments that provide good mixing of sample and extractant may be applied alternatively.

Transfer after phase separation an aliquot of the *n*-hexane phase into a suitable autosampler vial and analyse by UHPLC-APCI-MS/MS in selected reaction monitoring mode. Prevent contact of *n*-hexane with the PTFE lined septum by covering the vial's opening with aluminium foil prior to capping.

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 2000 µL of methanol (5.3) spiked with 50 µL of stock standard solution of DEHP-d4 in methanol (6.5). 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 (6.5). This sample is then extracted with 2000 µL of *n*-hexane (5.4) as described before (see 7.1) and analysed by UHPLC-APCI-MS/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 UHPLC-APCI-MS/MS DETERMINATION

7.3.1 UHPLC-APCI-MS/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[1].

[1] Chromatographic conditions and MS/MS parameters presented in this method description were optimised using a Waters Acquity UPLC system and a Waters Micromass Quattro Premier mass spectrometer. Instrument parameters and typical chromatograms are presented in Annex 1 and 2. The specified instrumental parameters remain indicative and should be optimised for the local instrumentation.

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

UHPLC parameters[1]	
UHPLC Column	Acquity BEH phenyl, 150 mm x 1.0 mm i.d., 1.7 µm particle size
Eluents	Methanol (A) Water (B)
Flowrate	100 µL/min
Gradient program	50:50 (A:B)/0 min – 78:22 (A:B)/2.25 min – 78:22 (A:B)/3.75 min – 100:0 (A:B)/5.25 min – 100:0 (A:B)/6.75 min – 50:50 (A:B)/7.00 min – 50:50 (A:B)/10.00 min
Column temperature	45 °C
Injection volume	2.0 µL
Needle wash	200 µL *n*-hexane, 600 µL methanol
Sample temperature	25 °C
Total run time	10.00 min
MS/MS parameters [1]	
Ionisation	Atmopsheric Pressure Chemical Ionisation
Ion polarity	positive
Operation mode	Selected reaction monitoring (SRM)
Nominal masses of recorded ion transitions (dwell time)	m/z 391 to 149 (0.167 s), 391 to 167 (0.167 s), 395 to 153 (0.167 s)

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 enter the chromatographic system via the solvents or might also be released from some LC parts (e.g. tubing).

Therefore the solvents and the UHPLC-MS/MS system should be inspected at the beginning of each analysis sequence for their 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 co-elution of the DEHP-d4 and DEHP peak and by comparison of the peak ratios for the DEHP quantifier transition m/z 391 to 149, and the DEHP qualifier transition m/z 391 to 167 from sample extracts and standard solutions. The ratios for sample extracts 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 transition 391 to 149)

$A_{DEHP\text{-}d4}$ is the area of the DEHP-d4 peak (of m/z transition 395 to 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 transition 391 to 149 of the test sample

$A_{DEHP\text{-}d4}$ is the area of the DEHP-d4 peak corresponding to peak of of m/z transition 395 to 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 (6.4) 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 0.99 and ordinary least squares linear regression functions were linear based on the Mandel Test. Matrix effects were not found. Therefore instrument calibration can be performed with standards in *n*-hexane.

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: 5.2 %

DEHP concentration level 50 mg/L: 1.6 %

DEHP concentration level 100 mg/L: 1.8 %

Intermediate precision was estimated from analysis on three different days 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 three different days by two different operators. The analyses were carried out on these different days by applying different positive displacement pipettes and different standard solutions of DEHP-d4 in methanol.

The intermediate precision relative standard deviation were at

DEHP concentration level 5 mg/L: 7.6 %

DEHP concentration level 50 mg/L: 7.1 %

DEHP concentration level 100 mg/L: 3.6 %

10.3 LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

Under the current instrumental conditions the limit of detection (LOD) and limit of quantification (LOQ) for DEHP in sports drinks can be extrapolated from the signal-to-noise (S/N) ratios obtained for the responses in the m/z transition 391 to 149. The limit of detection (S/N = 3) was 0.25 mg/L. The limit of quantification (S/N = 10) was approximately 1.0 mg/L.

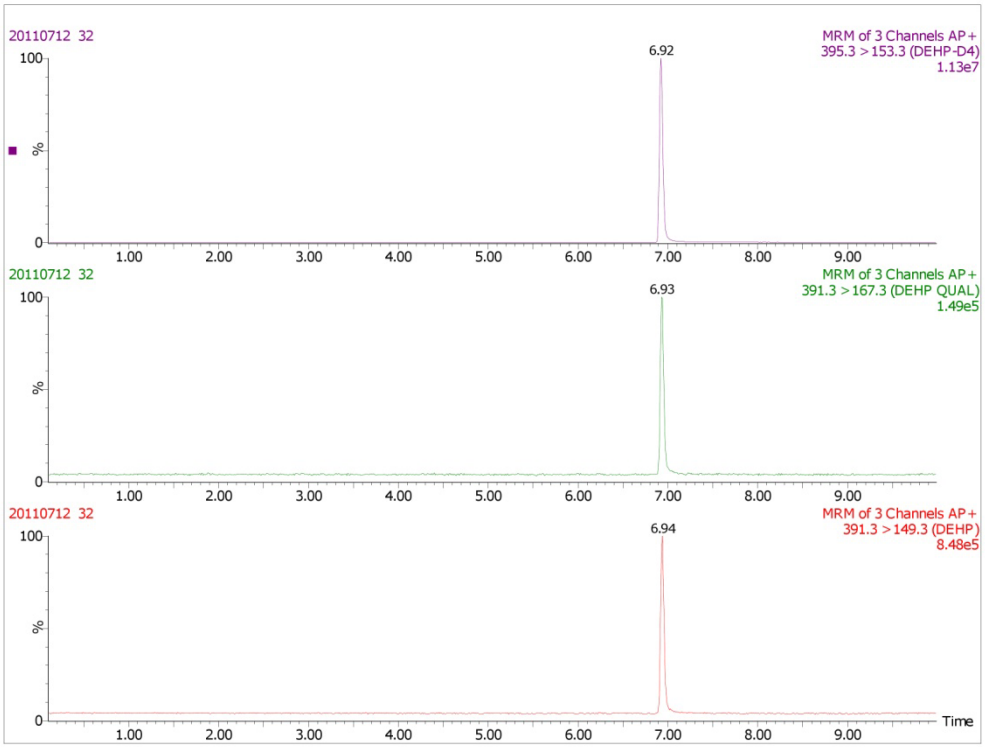
10.4 RECOVERY

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 100 ± 6% .

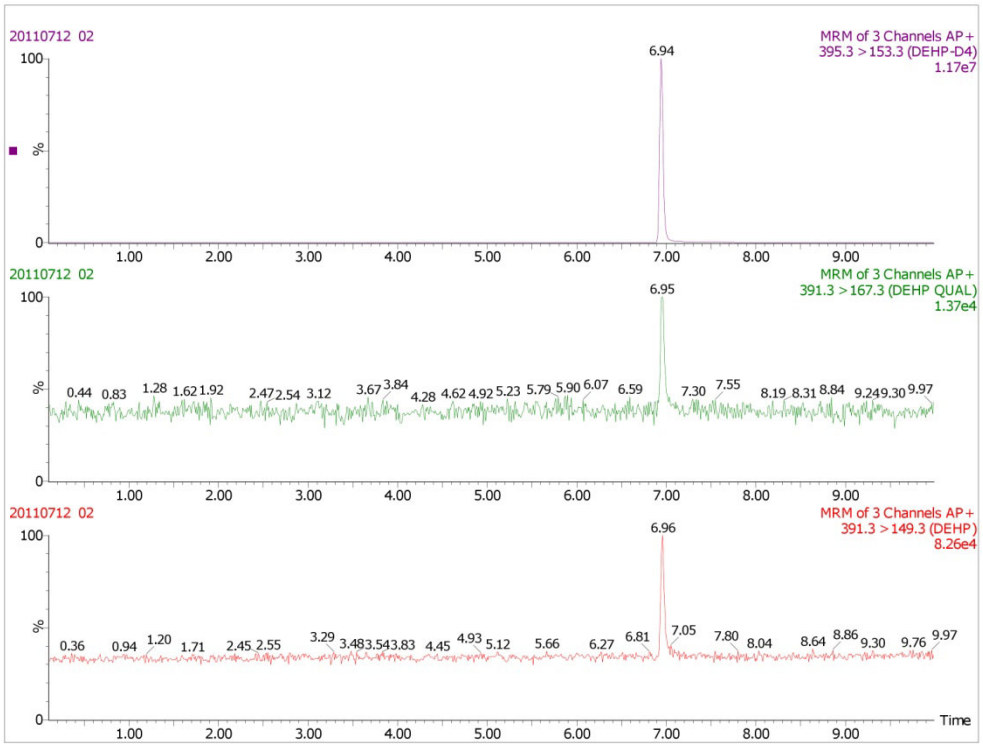
ANNEX 1

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

a)



b)



ANNEX 2

Example of instrumental settings used during method development for a Waters Micromass Quattro Premier mass spectrometer

---------- MS Instrument Parameters ----------
Ionization Mode API+
Calibration Static 2
Corona (µA) 1.50
Current Mode
Cone (V) 20
Extractor (V) 3
RF Lens (V) 0.0
Source Temperature (°C) 125
Desolvation Temp (°C) 400
Cone Gas Flow (L/h) 10
Desolvation Gas Flow (L/h) 250
LM 1 Resolution 15.0
HM 1 Resolution 15.0
Ion Energy 1 1.0
Entrance 0
Collision 18
Exit 1.0
LM 2 Resolution 15.0
HM 2 Resolution 15.0
Ion Energy 2 1.0
Multiplier (V) 650
Syringe Pump Flow (µL/min) 0
Pressure (m bar) 3.95e-3
Collision Gas Flow (mL/Min) 0.30
Source T-WAVE Parameters Automated
Collision Cell T-WAVE Parameters Automated

---------- MS Scan Parameters ----------
Cycle time (secs): 0.516
Inter Scan Delay (secs): 0.005
Inter Channel Delay (secs):0.005
Span (Da): 1.00

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