Application Note

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Quantification of Monohexyl Phthalate in Human Urine

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Abstract

Recent studies have highlighted the presence of monohexyl phthalate (MnHexP), a degradation product of certain plasticizers, in human urine samples. This application note describes the development and validation of a sensitive and precise UHPLC method for the quantification of MnHexP in urine. The method uses an Agilent 1290 Infinity III LC system coupled with an Agilent 6475 triple quadrupole LC/MS (LC/TQ), operating in multiple reaction monitoring (MRM) mode.

Human urine samples were prepared by dilution with acetonitrile (ACN), followed by chromatographic separation using an Agilent InfinityLab Poroshell HPH-C18 column. The method demonstrated excellent linearity ($R^2 \geq 0.99$) over a concentration range of 0.02 to 100 ng/mL. The instrument limit of detection (LOD) was 0.02 ng/mL for the quantifier ion transition (*m/z* 251.1 → 148.9). Method precision, assessed through recovery repeatability (RSDr), was 2.1%.

The developed method effectively separates MnHexP from matrix interferences, providing a reliable tool for monitoring phthalate exposure in human populations. The findings underscore the importance of continuous surveillance of MnHexP levels to assess potential health risks associated with phthalate exposure.

Introduction

Recent studies have raised concerns about the presence of MnHexP, a degradation product of certain plasticizers, in urine samples. The state agency for Nature, Environment, Climate & Consumer Protection of North Rhine-Westphalia (LANUV) detected MnHexP in follow-up analyses of children's urine samples. This substance is potentially a metabolite from various phthalates or could be ingested directly as hexyl hydrogen phthalate.[1]

Phthalates, commonly used as plasticizers in plastics such as polyvinyl chloride (PVC), are not firmly bound and can be released into the environment, leading to widespread detection in soil, water, and air. The human biomonitoring study by LANUV showed an increase in MnHexP detection from 26% in 2017/18 to 61% in 2020/21 urine samples, with concentrations rising from 0.28 to 2.09 µg/L.[2]

The Federal Environment Agency also found MnHexP in more than a third of adult urine samples in preliminary evaluations of the sixth German Environmental Health Study.[1] While the presence of MnHexP indicates exposure, it does not necessarily imply an immediate health risk. However, due to the reprotoxic nature of MnHexP and its precursors, efforts are being made to minimize intake of these substances.[1,2]

Experimental

Chemicals and reagents

Agilent InfinityLab LC/MS-grade acetonitrile (ACN) methanol (MeOH), water, and ammonium acetate were used for the study. Ammonium acetate was purchased from VWR International GmbH, (Darmstadt, Germany).

Standards and solutions

A single standard of monohexyl phthalate (CAS Number 24539-57-9) was purchased from LGC Standards GmbH (Wesel, Germany).

The neat compound was weighed, dissolved in ACN, and stored in a refrigerator at 4 °C if not used immediately.

An intermediate standard mix (mix 1) at a concentration of 1,000 µg/L was prepared in ACN from stock standards and used for the rest of the experiments. Mix 1 was used for the preparation of the matrix-matched calibration curve.

Sample preparation

Human urine was taken and diluted with ACN.

The following supplies were used for sample preparation:

- A vortex mixer (VWR International GmbH, Darmstadt, Germany)
- A 500 µL sample of neat ACN was mixed with a 500 µL of urine sample. After spiking, the samples were capped tightly and vortexed.
- Matrix-matched calibration standards (postspiked standards) were prepared and used to assess the performance of the conducted workflow. Unfortified human urine was used as a blank.
- Matrix-matched calibration standards were prepared. Serial dilutions were created from mix 1 to prepare 12 calibration concentration levels of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 µg/L. Calibration standards were freshly prepared and stored in a refrigerator at 4 °C if not used immediately.

Instrumentation

For chromatographic separation, an InfinityLab Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm column (part number 695775-702) installed on a 1290 Infinity III LC system was used. An Agilent InfinityLab PFC delay column, 4.6 × 30 mm (part number 5062-8100) was used to delay possible background signals.

The individual modules of the 1290 Infinity III LC system included:

- Agilent 1290 Infinity III high-speed pump (G7120A)
- Agilent 1290 Infinity III multisampler (G7167B)
- Agilent 1290 Infinity III multicolumn thermostat column compartment (G7116B)

The LC system conditions are listed in Table 1.

A 6475 LC/TQ with an Agilent Jet Stream technology ion source (AJS) was operated in MRM mode (Table 3). Data acquisition and processing were performed using Agilent MassHunter software, version 12.2. The 6475 LC/TQ parameters are shown in Table 2.

Table 1. LC method parameters.

Parameter	Value
Column	Agilent InfinityLab Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm column (p/n 695775-702)
Column Temperature	40 °C
Injection Volume	1 µL
Autosampler Temperature	8 °C
Mobile Phase A	2 mM Ammonium acetate in water
Mobile Phase B	MeOH
Flow Rate	0.4 mL/min
Gradient	Time (min) %A %B 0 95 5 1 95 5 7 0 95 9 0 95 9.01 95 5
Postrun Time	2 minutes
Needle Wash	Step Time (s) Solvent 1 5 ACN Seat backflush and needle wash 2 5 MeOH Seat backflush and needle wash 3 5 ACN:MeOH 40:60 (v:v) Seat backflush and needle wash

Table 2. Agilent 6475 LC/TQ instrument parameters.

Parameter	Value
Ionization Mode	Positive ESI with Agilent Jet Stream Technology ion source (AJS)
Acquisition Type	MRM
Cycle Time	0.4 s
Stop Time	11.01 min
Gas Temperature	250 °C
Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,000 V (+)
Nozzle Voltage	0 V

Table 3. MS method parameters.

Compound	Precursor Ion	Product Ion	Dwell Time	Fragmentor Voltage	Capillary Electrophoresis (V)	Polarity
MnHexP	251.1	148.9	100	80	8	Positive
MnHexP	251.1	120.9	100	80	34	Positive
MnHexP	251.1	92.9	100	80	40	Positive
MnHexP	251.1	64.9	100	80	40	Positive

Results and discussion

Development of the UHPLC method

A major focus of this work was to develop a suitable UHPLC method to separate the analyte from matrix interference. Figure 1 illustrates the interference for the qualifier ion transition resulting from the urine matrix.

Figure 2 demonstrates the sensitivity of the quantifier ion transition *m/z* 251.1 → 148.9 with a signal-to-noise (S/N) value of 20 (peak-to-peak, noise region 5.5 to 5.6 minutes).

Verification of workflow performance

The workflow performance was evaluated based on the criteria of linearity, method sensitivity, and precision. The batch included solvent blank, matrix-matched calibration standards, and matrix blank.

Linearity

Matrix-matched standards of mix 1 were used to generate calibration curves ranging from 0.02 to 100 ng/mL, using 12 calibration points. The following regression model was used for the calibration of the linearity response function: Linear, Origin: Ignore, Weight: 1/x. The target compound met the calibration curve linearity requirement of $R^2 \geq 0.99$.

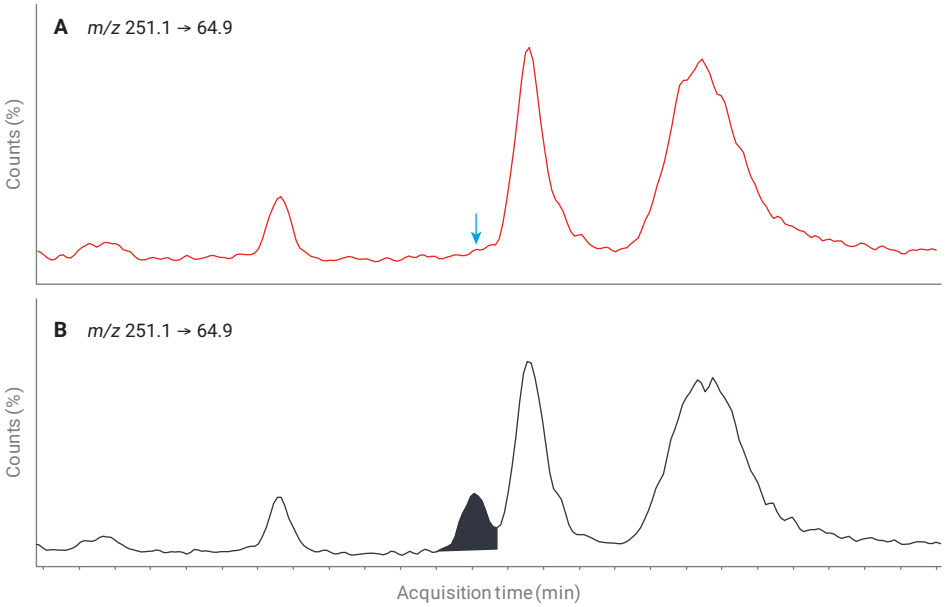

Figure 1. Stacked chromatograms for the qualifier ion transition (*m/z* 251.1 → 64.9) of a blank urine sample diluted with 500 µL of acetonitrile (A) and a spiked urine sample at 400 ng/L diluted with 500 µL of acetonitrile (B). Results are from 200 ng/L in-vial concentration (0.2 pg on column).

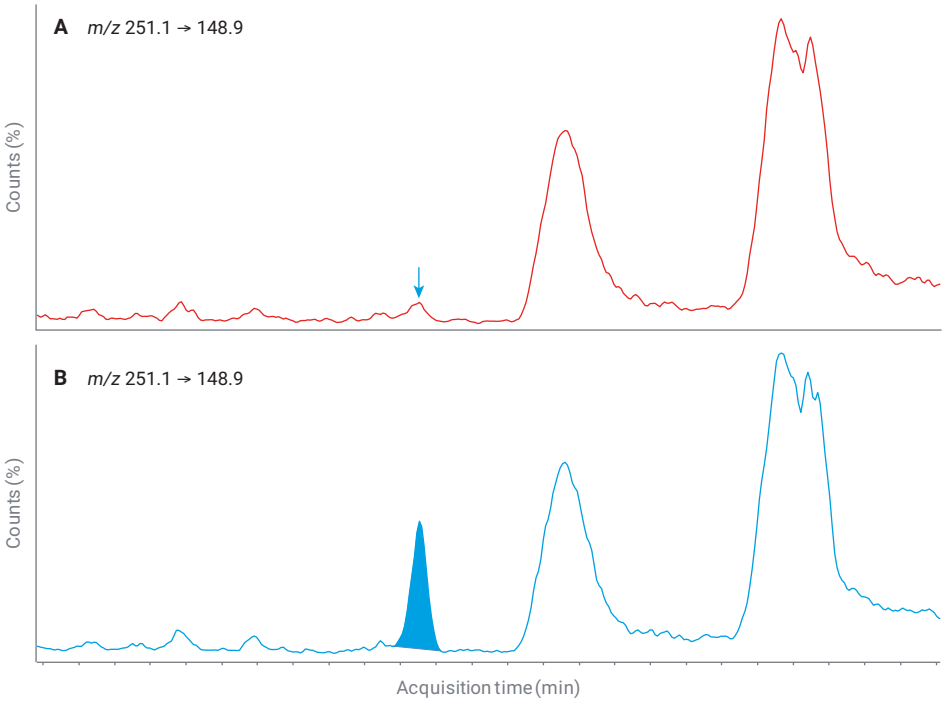

Figure 2. Stacked chromatograms for the quantifier ion transition (*m/z* 251.1 → 148.9) of a blank urine sample diluted with 500 µL of acetonitrile (A), and a spiked urine sample at 200 ng/L diluted with 500 µL of acetonitrile (B). Results are from 100 ng/L in-vial concentration (0.1 pg on column).

Instrument limit of detection

For the application of MnHexP analysis in a regulated field, it is crucial to implement a sensitive workflow. Therefore, the instrument LOD was used to evaluate the method sensitivity. The LOD was established based on matrix-matched calibration standards for an S/N ≥ 10. The S/N was obtained using the peak height and peak-to-peak algorithm embedded in Agilent MassHunter Quantitative Analysis software. The timeframe for the noise region was manually chosen and had a length of 0.1 minutes (0.1 minutes before and after the chromatographic peak).

MnHexP showed an instrument LOD of 0.02 ng/mL for the quantifier ion (Figure 3). These results demonstrate the sensitivity of the 6475 LC/TQ for a urine matrix.

Method precision

The method precision was determined by use of RSDr based on the variation of recovery values from replicates of prespiked QC samples spiked at 200 ng/L. The RSDr was determined by calculation of percent relative standard deviation (%RSD) of recovery using 10 injections. The RSDr accounts to 2.1%, highlighting the high repeatability of the instrument. Example chromatograms of the 10 replicates for MnHexP are given in Figure 4.

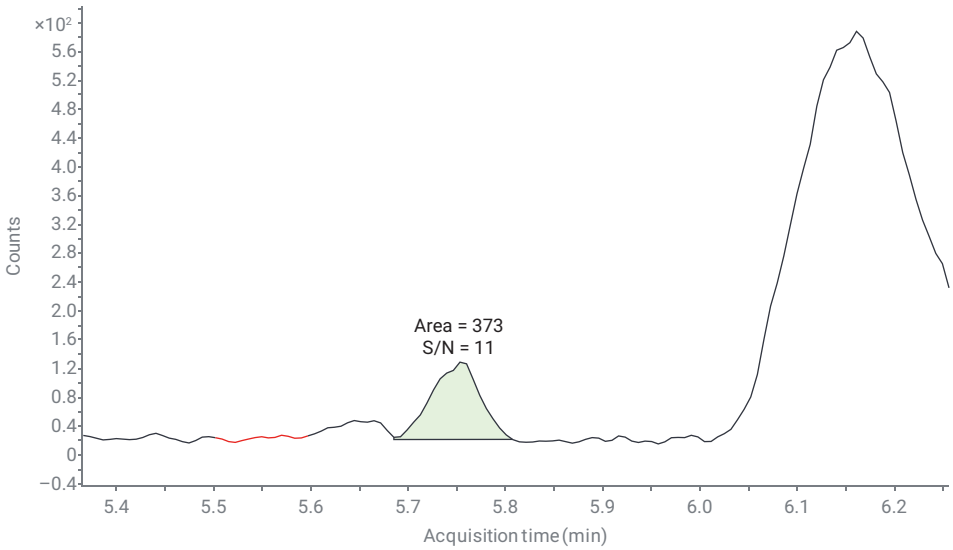

Figure 3. Chromatogram for the quantifier ion transition (*m/z* 251.1 → 148.9) of a spiked urine sample at 20 ng/L diluted with 500 µL of acetonitrile (20 pg on column).

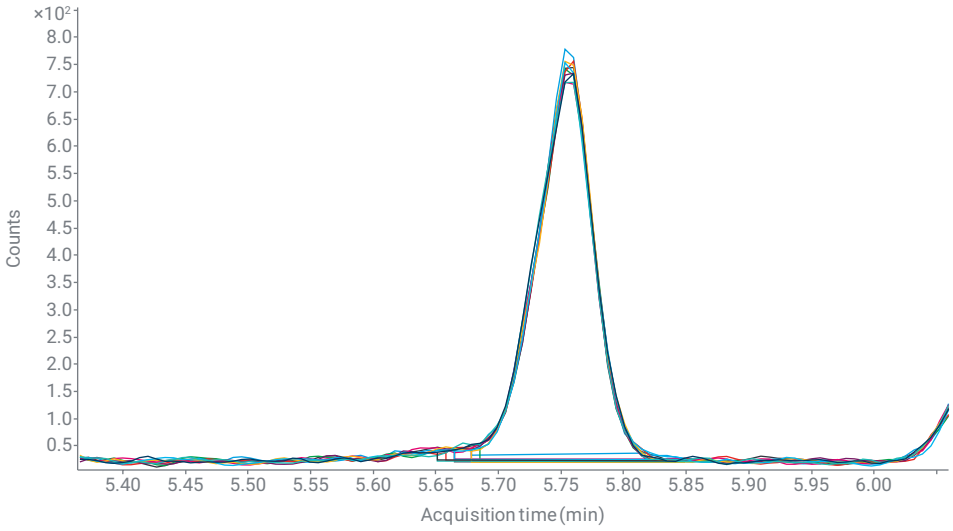

Figure 4. Overlaid chromatograms for the quantifier ion transition (*m/z* 251.1 → 148.9) for MnHexP, acquired from 10 injections.

Conclusion

The UHPLC method developed for the quantification of MnHexP in human urine demonstrated high sensitivity, precision, and linearity. The method effectively separated MnHexP from matrix interferences, as evidenced by the clear chromatographic peaks and high signal-to-noise ratios. The instrument LOD was established at 0.02 ng/mL, showcasing the method's capability to detect low concentrations of MnHexP in urine samples.

The precision of the method, indicated by an RSDr of 2.1% for recovery values, underscores its reliability for routine analysis. The increase in MnHexP detection in recent human biomonitoring studies highlights the importance of continuous monitoring and assessment of phthalate exposure in the population.

Overall, this method provides a robust and reliable approach for the quantification of MnHexP in human urine, contributing valuable data for environmental and health risk assessments.

References

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