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4-*tert*-Octylphenol and *p*-nonylphenol – Determination of 4-*tert*-octylphenol and *p*-nonylphenol in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2019

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Biomonitoring Methods

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and validated the presented biomonitoring method. The analytical method described hereinafter permits the selective detection of 4-*tert*-octylphenol as well as the sum of various branched *p*-nonylphenol isomers in urine. After adding the labelled internal standards ($^{13}C_6$-4-*tert*-octylphenol and $^{13}C_6$-*p*-nonylphenol), the samples are enzymatically hydrolysed to release the analytes from the conjugated alkylphenols. After online SPE, the analytes are separated by liquid chromatography and analysed using tandem mass spectrometry. A quantitation limit of 2 µg/L each is obtained for the analytes. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed.

Keywords

alkylphenols; 4-tert-octylphenol; p-nonylphenol; urine; biomonitoring; Analyses in Biological Materials; LC-MS/MS; tandem mass spectrometry; online-SPE

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4-*tert*-Octylphenol and *p*-nonylphenol – Determination of 4-*tert*-octylphenol and *p*-nonylphenol in urine by LC-MS/MS

Matrix:	Urine
Hazardous substances:	4-*tert*-Octylphenol and *p*-nonylphenol (branched, technical grade)
Analytical principle:	High-performance liquid chromatography coupled with tandem mass spectrometric detection (LC-MS/MS)
Completed in:	October 2015

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
4-*tert*-Octylphenol (4-(1,1,3,3-Tetramethylbutyl)phenol)	140-66-9	4-*tert*-Octylphenol	140-66-9
p-Nonylphenol (branched, technical grade)	84852-15-3 25154-52-3	*p*-Nonylphenol (branched)	84852-15-3 25154-52-3

Summary

The analytical method described hereinafter permits the selective detection of 4-*tert*-octylphenol as well as the sum of various branched *p*-nonylphenol isomers in urine. After adding the labelled internal standards ($^{13}C_6$-4-*tert*-octylphenol and $^{13}C_6$-*p*-nonylphenol), the samples are enzymatically hydrolysed to release the analytes from the conjugated alkylphenols. After online SPE, the analytes are separated by liquid chromatography and analysed using tandem mass spectrometry. A quantitation limit of 2 µg/L each is obtained for the analytes. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed.

Reliability data of the method

4-*tert*-Octylphenol

Within-day precision:	Standard deviation (rel.)	s_w = 7.0%, 4.4% or 2.5%
	Prognostic range	u = 15.8%, 10.0% or 5.6%
	at a spiked concentration of 2 µg, 10 µg or 50 µg 4-*tert*-octylphenol per litre urine and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 10.5%, 5.5% or 3.2%
	Prognostic range	u = 22.5%, 11.8% or 6.9%
	at a spiked concentration of 1 µg, 5 µg or 25 µg 4-*tert*-octylphenol per litre urine and where n = 15 determinations	
Accuracy:	Recovery rate (rel.)	r = 104%, 98% or 98%
	at a spiked concentration of 2 µg, 10 µg or 50 µg 4-*tert*-octylphenol per litre urine and where n = 10 determinations	
Detection limit:	0.6 µg 4-*tert*-octylphenol per litre urine	
Quantitation limit:	2.0 µg 4-*tert*-octylphenol per litre urine	

p-Nonylphenol (branched)

Within-day precision:	Standard deviation (rel.)	s_w = 4.4%, 3.2% or 4.1%
	Prognostic range	u = 10.0%, 7.2% or 9.3%
	at a spiked concentration of 2 µg, 10 µg or 50 µg *p*-nonylphenol per litre urine and where n = 10 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 4.8%, 5.2% or 3.8%
	Prognostic range	u = 10.3%, 11.2% or 8.2%
	at a spiked concentration of 1 µg, 5 µg or 25 µg *p*-nonylphenol per litre urine and where n = 15 determinations	
Accuracy:	Recovery rate (rel.)	r = 106%, 101% or 99%
	at a spiked concentration of 2 µg, 10 µg or 50 µg *p*-nonylphenol per litre urine and where n = 10 determinations	
Detection limit:	1.0 µg *p*-nonylphenol per litre urine	
Quantitation limit:	2.0 µg *p*-nonylphenol per litre urine	

General information on the hazardous substances

The compounds 4-*tert*-octylphenol and *p*-nonylphenol (branched) belong to the group of long-chain alkylphenols.

Technical grade 4-*tert*-octylphenol is a C8-alkylphenol, which consists to more than 95% of 4-(1,1,3,3-tetramethylbutyl)phenol (see Figure 1) and is mainly used in

the production of polymers (octylphenol formaldehyde resins). Octylphenol formaldehyde resins, in turn, are mainly used as rubber additive in tyre production. Besides, 4-*tert*-octylphenol is also used in the production of a group of surfactants, namely octylphenol ethoxylates. The resins and ethoxylates produced from 4-*tert*-octylphenol are then used, for example, in paints, adhesives and tyres [OECD 1994; UBA 2018 b].

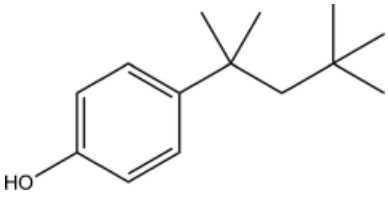


Figure 1 Structure of 4-*tert*-octylphenol (4-(1,1,3,3-tetramethylbutyl)phenol).

Due to the technical production process, the C9-alkylphenol *p*-nonylphenol (branched) occurs as a complex mixture of isomers. Figure 2 shows a typical GC-HRMS chromatogram of the *p*-nonylphenol standard used for this method, which was obtained during method development.

Wheeler et al. [1997] were able to identify a total of 22 *p*-nonylphenol isomers using high-resolution GC-MS. It was found that this mixture consists of ten isomers of the α-dimethyl type (48.6%), four isomers of the α-methyl, α-ethyl type (24.7%), three isomers of the α-methyl, β-methyl type (8.9%), two isomers of the α-methyl type (6.6%) and three isomers of the α-methyl, α-propyl type (11.2%). Figure 3 shows some examples of isomeric structures.

p-Nonylphenol is primarily used as an intermediate in the manufacture of nonylphenol ethoxylates and as a monomer in the production of phenolic resins [ECHA 2018 a; UBA 2018 a].

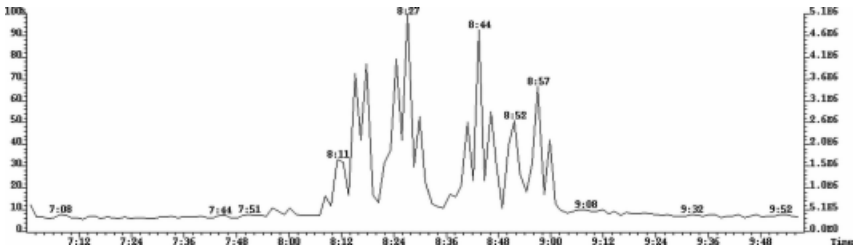
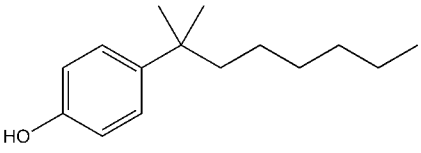
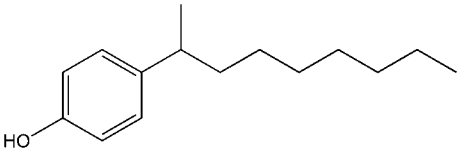


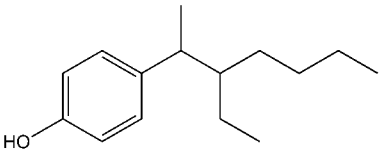
Figure 2 GC-HRMS chromatogram of a *p*-nonylphenol standard solution.



4-(1,1-Dimethylheptyl)phenol



4-(1-Methyloctyl)phenol



4-(1-Methyl-2-ethylhexyl)phenol

Figure 3 Examples of the structures of technical grade *p*-nonylphenol isomers.

4-*tert*-Octylphenol and *p*-nonylphenol have been identified as endocrine disruptors and have been shown to disrupt the endocrine system in fish. The use of *p*-nonylphenol in applications with direct release into the environment has therefore been severely restricted in the European Union since 2003 [Gabriel et al. 2008; UBA 2018 a]. 4-*tert*-Octylphenol and *p*-nonylphenol have been included in the candidate list of substances of very high concern (SVHC) under the REACH Regulation due to the observed endocrine disrupting effects in fish [ECHA 2018 b; UBA 2018 a, b]. For details on the toxicological evaluation of 4-*tert*-octylphenol, please refer to the respective MAK Value Documentations [Hartwig 2016; Hartwig 2017]. *p*-Nonylphenol has not been evaluated by the Commission yet.

Alkylphenol ethoxylates and their degradation products are still detected both in aquatic sediments in the environment [UBA 2018 a] and in drinking water [Wang and Schnute 2010]. It is assumed that these substances are continuously released into the environment due to washing of imported textiles and indirectly through further applications [OECD 1994; UBA 2018 a].

Octylphenol ethoxylates and nonylphenol ethoxylates may be degraded in the environment to alkylphenols as intermediary substances, in particular under anaerobic conditions. Both alkylphenols, however, are inherently biodegradable [OECD 1994]. Data on the oral bioavailability of octylphenol and nonylphenol in animal studies vary widely in the literature, ranging from 2 to 40%. In animal studies, the elimina-

tion half-life is given to be in the range of a few hours [Hartwig 2016]. In humans, 4-*tert*-octylphenol and *p*-nonylphenol can be detected in the urine of the general population [Calafat et al. 2005, 2008; Inoue et al. 2003; Jing et al. 2011], suggesting that both compounds are present ubiquitously in the environment.

In-vitro studies and animal experiments suggest that alkylphenols are glucuronidated in the body and then excreted via bile or urine [Nomura et al. 2008; Ye et al. 2007; Zalko et al. 2003]. An animal study on rats has shown that 20–50% of the dose is excreted unchanged or as glucuronide in urine within seven days after oral administration of nonylphenol [Müller and Certa 2013].

Contents

1 General principles

The analytical method described hereinafter permits the selective detection of 4-*tert*-octylphenol as well as the sum of various branched *p*-nonylphenol isomers in urine. After adding the labelled internal standards ($^{13}C_6$-4-*tert*-octylphenol and $^{13}C_6$-*p*-nonylphenol), the samples are enzymatically hydrolysed to release the analytes from the conjugated alkylphenols. After online SPE, the analytes are separated by liquid chromatography and analysed using tandem mass spectrometry. A quantitation limit of 2 µg/L each is obtained for the analytes. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed.

2 Equipment, chemicals and solutions

2.1 Equipment

- LC-MS/MS system consisting of an HPLC system with a gradient pump, an eluent degasser, a column thermostat and an autosampler (e.g. Waters Alliance LC) as well as a tandem mass spectrometric detector (e.g. Waters Quattro Ultima Tandem MS)
- LC column and trap column: Zorbax Eclipse XDB-C8 5 µm, 4.6 × 50 mm (e.g. Agilent, No. 946975-906)
- Preconcentration column: Oasis HLB 25 µm, 2.1 × 20 mm (e.g. Waters, No. 186002036)
- Laboratory shaker (e.g. IKA Vibrax VXR)
- Analytical balance (e.g. Sartorius)
- Incubator (e.g. Binder)
- Various volumetric flasks and beakers (e.g. Schott)
- 12 mL test tubes with Teflon-coated screw caps (e.g. Schütt)
- Various pipettes and Multipettes® (e.g. Eppendorf)
- 2 mL Crimp vials with caps (e.g. Waters)
- pH meter (e.g. Mettler Toledo)
- Glass containers for urine collection (e.g. Duran)

2.2 Chemicals

Unless otherwise specified, all chemicals must be at least p. a. grade.

- Acetonitrile (e.g. Merck, No. 1.00017)
- Ultrapure water (e.g. Milli-Q water)
- *β*-Glucuronidase (e.g. Roche, No. 03707598001)
- Acetic acid (glacial acetic acid), 100% (e.g. Merck, No. 1.00066)
- Sodium acetate, anhydrous (e.g. Merck, 1.062464)
- 4-*tert*-Octylphenol technical grade, 98.06% (e.g. SI-Group)
- *p*-Nonylphenol technical grade, 90.1% (e.g. SI-Group)
- $^{13}C_6$-4-*tert*-Octylphenol (ring-$^{13}C_6$), 10 µg/mL in acetone, (e.g. Sigma-Aldrich, No. 33565)

- $^{13}C_6$-*p*-Nonylphenol (3,6,3-nonylphenol ring-$^{13}C_6$), 10 µg/mL in acetone, (e.g. Sigma-Aldrich, No. 33574)

2.3 Solutions

- 0.1 M Sodium acetate buffer
  Exactly 4.1 g sodium acetate are weighed into a 400 mL beaker and dissolved in approximately 250 mL ultrapure water. Then, the pH is adjusted to pH 5 (pH meter) with glacial acetic acid and the solution is quantitatively transferred to a 500 mL volumetric flask. The flask is then made up to the mark with ultrapure water.

When stored in the refrigerator at +4 °C, the solution is stable for at least one month.

2.4 Internal standard (ISTD)

- ISTD spiking solution (1 mg/L)
  1 mL each of the standard solutions of $^{13}C_6$-4-*tert*-octylphenol and $^{13}C_6$-*p*-nonylphenol are pipetted into a 10 mL volumetric flask, which is then made up to the mark with acetonitrile.

When stored in the refrigerator at +4 °C, the ISTD spiking solution is stable for at least six months.

2.5 Calibration standards

- Stock solution I (1000 mg/L)
  Exactly 10 mg of 4-*tert*-octylphenol and 10 mg of branched *p*-nonylphenol are weighed into a 10 mL volumetric flask each and dissolved in acetonitrile. The two flasks are then filled to the mark with acetonitrile.
- Stock solution II (10 mg/L)
  100 µL each of stock solution I are pipetted into a 10 mL volumetric flask, which is then made up to the mark with acetonitrile.
- Spiking solution I (1 mg/L)
  1 mL of stock solution II is pipetted into a 10 mL volumetric flask, which is then made up to the mark with acetonitrile.
- Spiking solution II (0.1 mg/L)
  100 µL of stock solution II are pipetted into a 10 mL volumetric flask, which is then made up to the mark with acetonitrile.
- Spiking solution III (0.01 mg/L)
  100 µL of spiking solution I are pipetted into a 10 mL volumetric flask, which is then made up to the mark with acetonitrile.

When stored in the refrigerator at +4 °C, the stock solutions and spiking solutions are stable for at least six months.

The calibration standards are prepared in pooled urine from individuals not occupationally exposed to alkylphenols. To prepare the pooled urine, spot urine samples are collected in a suitable container, thoroughly mixed and stored at –20 °C until preparation of the standards and the control material.

Calibration standards in a concentration range between 0.5 and 100 µg 4-*tert*-octylphenol or *p*-nonylphenol per litre urine are prepared by diluting the spiking solutions with pooled urine each according to the pipetting scheme shown in Table 1. Additionally, the pooled urine used is included as a blank.

Table 1 Pipetting scheme for the preparation of calibration standards used to determine 4-*tert*-octylphenol and branched *p*-nonylphenols in urine.

Calibration standard	Spiking solution	Volume of the spiking solution	Volume of the pooled urine	Concentration of the analytes
		[µL]	[µL]	[µg/L]
Blank	–	–	500	–
1	III	25	475	0.5
2	II	5	495	1.0
3	II	10	490	2.0
4	II	25	475	5.0
5	I	5	495	10
6	I	10	490	20
7	I	25	475	50
8	I	50	450	100

3 Specimen collection and sample preparation

3.1 Specimen collection

The urine samples are collected in a suitable urine collection container made of glass (see also Section 9.4), aliquoted and stored at –20 °C until analysis.

3.2 Sample preparation

Prior to analysis, the urine samples are thawed at room temperature and mixed thoroughly. For sample preparation, 0.5 mL of the urine sample are transferred into a 2 mL crimp vial and 10 µL of the ISTD spiking solution as well as 1 mL of the sodium acetate buffer are added. The sample is thoroughly mixed on a vortex mixer. For hydrolysis, 10 µL of β-glucuronidase are added to the sample, the vial is sealed and the solution is mixed thoroughly. The sample is incubated overnight (16 h) in the incubator at 37 °C. After cooling to room temperature, the sample is directly injected into the LC-MS/MS system for analysis.

4 Operational parameters

Analysis was performed using an HPLC system coupled with a tandem mass spectrometer.

4.1 High performance liquid chromatography

The HPLC system employed for method development and validation comprised only one pump. Column switching for online SPE was accomplished using two six-port-two-position valves and two T-pieces (see flow chart in Figures 4a and 4b). Obviously, it is also possible to perform online SPE using more advanced and complex LC setups, e.g. dual pump setups. Operational parameters such as gradient profile and switching times must be optimised for each HPLC instrument and its specific online SPE setup.

Trap column:	Zorbax Eclipse XDB-C8 5 µm, 4.6 × 50 mm (installed upstream of the injection system)
Preconcentration column:	Oasis HLB 25 µm, 2.1 × 20 mm
Analytical column:	Zorbax Eclipse XDB-C8 5 µm, 4.6 × 50 mm
Mobile phase:	Mobile phase A: ultrapure water Mobile phase B: acetonitrile
Flow rate:	0.3 mL/min
Column temperature:	30 °C
Injection volume:	100 µL
Column switching program:	0–3.5 min: flow via preconcentration column 3.5–18 min: flow via preconcentration column and analytical column 18–20 min: flow via preconcentration column
Gradient program:	see Table 2

Table 2 Gradient program for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenols in urine.

Time	Mobile phase A	Mobile phase B	Flow rate
[min]	[%]	[%]	[mL/min]
0	80	20	0.3
4.0	80	20	0.3
5.0	5	95	0.3
10.0	5	95	0.3
10.5	80	20	0.3
20.0	80	20	0.3

4.2 Tandem mass spectrometry

Ionisation mode:	negative electrospray ionisation (ESI^-)
Source temperature:	150 °C
Desolvation temperature:	500 °C
Detection mode:	Multi Reaction Monitoring (MRM)
Cone gas flow:	100 L/h
Desolvation gas flow:	650 L/h
Entrance potential:	−2 V
Collision energy:	22 V
Exit potential:	2 V
Multiplier:	650 V

All ion source settings and MRM parameters are instrument-specific and must be adjusted individually by the user. The parameters specified above are therefore intended as a rough guide only. All other parameters have to be optimised in accordance with the manufacturer's specifications.

5 Analytical determination

100 µL each of the samples prepared as described in Section 3 are injected into the LC-MS/MS system. Identification of the analytes is based on the retention times and specific mass transitions. The retention times of the analytes and internal standards as well as the ion traces used are presented in Table 3. Several reagent blank samples (ultrapure water instead of the urine sample) and at least two quality control samples (see Section 8) are included in each analytical run.

The retention times given in Table 3 are intended as a rough guide only and may differ depending on the HPLC instrument setup used. Users must ensure proper separation performance of the HPLC column used influencing the retention behaviour of the analytes. Figures 5 and 6 (in the Appendix) show, as an example, chromatograms of pooled urine samples spiked with the analytes at different concentration levels.

6 Calibration

The calibration standards described in Section 2.5 are prepared and processed in the same way as the samples (see Section 3) and analysed using LC-MS/MS (cf. Section 4). Calibration graphs are obtained by plotting the ratios of analyte peak area to internal standards peak area against the concentration of the calibration standards. Under the analytical conditions described, the calibration graphs are linear in the concentration range from 0.5 to 1000 µg/L. However, calibration up to 50 µg/L has proved to be sufficient for both 4-*tert*-octylphenol and *p*-nonylphenol. Based on the samples analysed, concentrations exceeding 50 µg/L are not expected. Additionally, quantification in the range of the limit of quantitation is improved when keeping the calibration range as tight as possible. Figures 7 and 8 (in the Appendix) show examples of calibration graphs of the analytes.

Table 3 Retention times and ion transitions used for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenols in urine.

Analyte	Retention time	Ion trace [*m*/*z*]	
	[min]	Q1	Q3
4-*tert*-Octylphenol	11.66	205.22	133.04
$^{13}C_6$-4-*tert*-Octylphenol	11.66	211.14	138.89
p-Nonylphenol	12.12	219.22	133.10
$^{13}C_6$-*p*-Nonylphenol	12.12	225.08	138.95

7 Calculation of the analytical results

The analyte concentrations in the urine samples are calculated using the calibration function of the respective analytical run (Section 6). To calculate the analyte concentration in a urine sample, the peak area of the respective analyte is divided by the peak area of the corresponding internal standard. The ratio thus obtained is entered in the calibration function (Section 6) to give the respective analyte concentration in µg/L. Any reagent blank values have to be subtracted from the analytical results.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundesärztekammer (German Medical Association) and in a general chapter of the MAK Collection for Occupational Health and Safety [Bader et al. 2010; Bundesärztekammer 2014]. To check precision, at least two quality control samples with known analyte concentrations are analysed within each analytical run. As material for quality control is not commercially available, it must be prepared in the laboratory. To this end, pooled urine from individuals occupationally not exposed to alkylphenols is used and spiked with the standard solutions of the analytes, so that the resulting analyte levels in the control material are within the relevant concentration range (e.g. 10 µg/L and 50 µg/L). Aliquots of these samples are stored at –20 °C and are included in each analytical run as quality control samples. The nominal value and the tolerance ranges of the quality control material are determined in the course of a preanalytical period (one analysis of the control material each on 10 different days) [Bader et al. 2010].

At the same time, at least three reagent blanks are included in each analytical run to identify potential interferences caused by the chemicals (cf. Section 9.4). Here, ultrapure water is used instead of urine.

9 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and validation of the method in a second, independent laboratory.

9.1 Precision

Within-day precision was determined using pooled urine samples from individuals not occupationally exposed to alkylphenols. The pooled urine was spiked with 2 µg, 10 µg or 50 µg/L each of 4-*tert*-octylphenol and *p*-nonylphenol, processed ten times in parallel and analysed. The obtained relative precision data are presented in Table 4.

Table 4 Within-day precision for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in urine (n = 10).

Analyte	Spiked concentration	Standard deviation (rel.) s_w	Prognostic range u
	[µg/L]	[%]	[%]
4-*tert*-Octylphenol	2	7.0	15.8
	10	4.4	10.0
	50	2.5	5.6
p-Nonylphenol (branched)	2	4.4	10.0
	10	3.2	7.2
	50	4.1	9.3

To determine day-to-day precision, pooled urine samples were spiked with 1 µg, 5 µg and 25 µg/L each of the analytes, processed on 15 different days and analysed. The obtained precision data are presented in Table 5.

Table 5 Day-to-day precision for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in urine (n = 15).

Analyte	Mean concentration	Standard deviation (rel.) s_w	Prognostic range u
	[µg/L]	[%]	[%]
4-*tert*-Octylphenol	1.2	10.5	22.5
	5.0	5.5	11.8
	24.7	3.2	6.9
p-Nonylphenol (branched)	1.1	4.8	10.3
	4.6	5.2	11.2
	24.2	3.8	8.2

9.2 Accuracy

Recovery experiments were performed to determine the accuracy of the method, based on the tests to determine within-day precision. The relative recovery rates thus obtained are presented in Table 6.

Table 6 Relative recovery rates for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in urine, derived from the within-day precision data (n = 10).

Analyte	Spiked concentration	Mean relative recovery *r*	Range
	[µg/L]	[%]	[%]
4-*tert*-Octylphenol	2	104	94–116
	10	98	92–106
	50	98	94–102
p-Nonylphenol (branched)	2	106	97–113
	10	101	96–107
	50	99	92–104

In order to determine the impact of different urine matrices, ten individual urine samples were spiked with 10 µg/L or 100 µg/L each of the analytes, processed and analysed. The results are presented in Table 7.

Table 7 Relative recovery rates for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in spiked individual urine samples (n = 10).

Analyte	Spiked conc.	Standard deviation (rel.) s_w	Prognostic range *u*	Mean relative recovery *r*	Range
	[µg/L]	[%]	[%]	[%]	[%]
4-*tert*-Octylphenol	10	5.3	11.8	103	92–111
	100	9.4	21.0	97	83–108
p-Nonylphenol (branched)	10	2.7	6.1	102	96–106
	100	2.9	6.7	102	98–102

9.3 Limits of detection and limits of quantitation

The limits of detection and limits of quantitation were determined based on the calibration curve method by use of the six lowest calibration standards. The values determined for both substances in urine are given in Table 8.

Table 8 Limits of detection and limits of quantitation for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in urine.

Analyte	Detection limit	Quantitation limit
	[µg/L]	[µg/L]
4-*tert*-Octylphenol	0.6	2.0*
p-Nonylphenol (branched)	1.0	2.0*

* depending on the reagent blank level (cf. Sections 9.4 and 10)

9.4 Sources of error

At the early stage of method development, liquid-liquid extraction was used to isolate both alkylphenols from the urine sample. However, due to a high reagent blank level of about 10 µg/L and low recovery rates of 20% and 35% for 4-*tert*-octylphenol and *p*-nonylphenol, respectively, this approach was abandoned. The use of an offline solid phase extraction was also tested, but was not a promising option either as the reagent blank level could not be reduced significantly.

This led to the implementation of the column switching technique with online sample enrichment and sample cleanup on a preconcentration column. After backflush of the sample onto the analytical column, chromatographic separation is performed followed by mass spectrometric detection in MRM mode. This technique allows a significant reduction of reagent blank values down to 2 µg/L and 3 µg/L for 4-*tert*-octylphenol and *p*-nonylphenol, respectively. The aim of the analytical method, however, was to achieve a limit of quantitation of about 1 µg/L. In numerous tests, thus, various sources of interferences/contamination were identified and appropriate measures for their reduction were developed.

The LC capillary system of the analytical instrument was identified as a possible source of blank values. As the analytes are easily adsorbed due to their lipophilic and acidic properties, there is a risk of accumulation in the capillaries and switching valves, in particular after the injection of larger amounts of analytes (e.g. after injection of calibration standards in the upper calibration range). This problem could be eliminated by subsequently injecting one or more aqueous samples to purge the system. As this problem may be attributable to the particular instrument used, it might not occur with other analytical systems.

The autosampler crimp vials and their caps presented another source of contamination. Comparative tests proved that the vials and caps described in Section 2.1 were ideal for analysing alkylphenols as they showed the lowest blank levels. Alternatively, it is also possible to rinse the vials with methanol prior to use and to use caps with Teflon septa. This measure also helps to avoid possible batch-related contamination and was consistently applied for later tests.

Deterioration of precolumns or analytical columns was also identified as a potential source of contamination. Here too, adsorbing sites may lead to a gradual accumulation of the analytes, which are then dissolved by the eluent and subsequently detected as "hot spots". Furthermore, traces of *p*-nonylphenol were found in various acetonitrile batches. These *p*-nonylphenol traces also accumulated in the downstream system and were sporadically released, leading to false positive results. This problem was identified only in detailed blank value studies. In order to avoid time-consuming batch tests, a trap column was installed upstream of the injection system to remove any *p*-nonylphenol traces from the eluents. The sum of all measures described above led to a reproducible blank value reduction to about 0.3 µg/L for 4-*tert*-octylphenol and 0.5 µg/L for *p*-nonylphenol.

Two different LC methods were tested. One method (described in the main section) is used for high sample throughput and is well suited for routine application. The second LC method leads to an improved matrix separation, but the retention times of the analytes are twice as high compared to the rapid method. Both LC variants yielded comparable precision data and may be used on a standard basis. For the alternative method, a different and longer analytical column as well as a modified gradient program were used. For details regarding the alternative method, please refer to Section 12.1 in the Appendix.

Potential contamination pathways must also be taken into account during sampling. As the two analytes may also diffuse from packaging material, the type and origin must be considered when choosing a urine collection container. We have successfully tested the use of glass containers with Teflon-coated caps or glass stoppers as well as of polypropylene containers, if these were rinsed with methanol prior to use. It is highly recommended to rinse all glassware used with methanol before starting the analysis, also including the screw cap vials used for storage and sample preparation. This also applies to glassware that has been freshly cleaned in a laboratory dishwasher and to glassware that has been cleaned and subsequently stored.

The issue with blank values was confirmed during external verification of the method, which was performed using an Agilent 1100 HPLC system coupled with an AB Sciex QTrap 5500 tandem mass spectrometer. Additionally, the buffer and *β*-glucuronidase used were identified as further sources of contamination, which made quantification in the range of the limit of quantitation difficult. Here too, the carryover effect after injection of samples with high analyte concentrations was observed, which could only be eliminated by subsequent injection of several blanks.

Various internal standard substances were tested in the course of method development. 4-*n*-Nonylphenol-d_4 and 4-*n*-nonylphenol-$^{13}C_6$ proved to be unsuitable as internal standards for branched *p*-nonylphenol as these could not fully compensate for analytical fluctuations (matrix and quenching) due to deviating physical properties (retention time, fragmentation). 4-*tert*-Octylphenol-$^{13}C_6$, which was used as an internal standard for 4-*tert*-octylphenol, also proved to be less than ideal as ISTD for *p*-nonylphenol. Reproducible analysis of branched *p*-nonylphenol was only possible by using *p*-nonylphenol-$^{13}C_6$ as ISTD.

10 Discussion of the method

The aim of the method development was a rapid, selective, sensitive and robust analytical method, that enables the simultaneous determination of both alkylphenols in a single analysis. The use of the LC-MS/MS technique made it possible to reduce the separation efficiency to such an extent that the complex *p*-nonylphenol isomer mixture eluted in a single peak. The mass transition used for analysis from *m/z* 219 to *m/z* 133 enables the detection of approximately 75% of all *p*-nonylphenol isomers. Preliminary tests have shown that unbranched 4-*n*-nonylphenol, which is often described in the literature, does not occur in the technical-grade *p*-nonylphenol standard (branched). Thus, analysis of this substance as a marker of general nonylphenol exposure cannot be deemed expedient.

Due to the ubiquitous occurrence and the physicochemical properties of 4-*tert*-octylphenol and *p*-nonylphenol a substantial blank value problem occurs through all steps of the procedure. This applies in particular to *p*-nonylphenol. Accordingly, the analytical method should involve as few steps as possible, since each additional analytical step also entails a risk of additional potential contamination. A reduction of the blank values to less than 1 µg/L was only possible by using the LC-MS/MS analysis and by using only one vial per sample for sample preparation.

Due to the blank value problem, it is necessary to test the suitability of the chemicals used and of the analytical system by running blank tests before starting alkylphenol analysis. This should be done by the analysis and documentation of at least three reagent blanks within each analytical run in addition to the analytical samples and the quality control samples. In compliance with the quality specifications of the used quality control samples, an analytical run can only be regarded as valid for the determination of background levels in the urine of the general population if the determined blank values are consistent and below 1 µg/L.

The use of $^{13}C_6$-labelled internal standards with similar fragmentation patterns compared to the analytes, allows the compensation of all analytical fluctuations, which is confirmed by the very good precision data. A higher deviation occurs only at the lowest spiked concentration, which can certainly be attributed to the already discussed blank value issues in the lower concentration range. Tests with spiked individual urine samples also show the robustness of the analytical method even when using different urine matrices.

To gain an insight into possible background exposure, urine samples from 40 individuals occupationally not exposed to alkylphenols were analysed. In two samples *p*-nonylphenol was found at a concentration level of 1.6 and 1.8 µg/L, respectively, whereas 4-*tert*-octylphenol could not be detected in any sample. These results clearly show that background exposure of the population is to be expected predominantly in the range of the limit of quantitation of this method.

The specific instructions given in Section 9.4 show the high demands for the development of a valid method for the determination of alkylphenols. This issue was also confirmed by the external experts during verification of this method and can thus be considered a major challenge for analysis of alkylphenols in the range of background levels. If the measures described in Section 9.4 for reducing the blank values are consistently adhered to, the sensitivity of the method may be further increased (to a limit of quantitation of about 1 µg/L).

Instruments used: LC-MS/MS system consisting of Waters Alliance HPLC system coupled with Waters Quattro Ultima MS tandem mass spectrometer.

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12 Appendix

12.1 Alternative analytical method

Trap column:	Zorbax Eclipse XDB-C8 5 µm, 4.6 × 50 mm
Preconcentration column:	Oasis HLB 25 µm, 2.1 × 20 mm
Analytical column:	Symmetry C8 3.5 µm, 2.1 × 150 mm
Mobile phase:	Mobile phase A: ultrapure water Mobile phase B: 1 mM ammonium acetate Mobile phase C: acetonitrile
Flow rate:	0.3 mL/min
Column temperature:	30 °C
Injection volume:	100 µL
Column switching program:	0–5 min: flow via preconcentration column 5–35 min: flow via preconcentration column and analytical column 35–40 min: flow via preconcentration column
Gradient program:	see Table 9

Due to the longer analytical column, the analytes elute much later, also leading to improved matrix separation (retention times for 4-*tert*-octylphenol and ISTD:

t_R = 26.3 min; *p*-nonylphenol and ISTD: t_R = 25.0 min). All other parameters remain unchanged with regard to the analytical method described in the main section.

Table 9 Alternative gradient program for the determination of 4-*tert*-octylphenol and branched *p*-nonylphenol in urine.

Time	Mobile phase A	Mobile phase B	Mobile phase C	Flow rate
[min]	[%]	[%]	[%]	[mL/min]
0	60	5	35	0.3
10.0	60	5	35	0.3
20.0	15	5	80	0.3
30.0	15	5	80	0.3
31.0	60	5	35	0.3
40.0	60	5	35	0.3

12.2 Figures

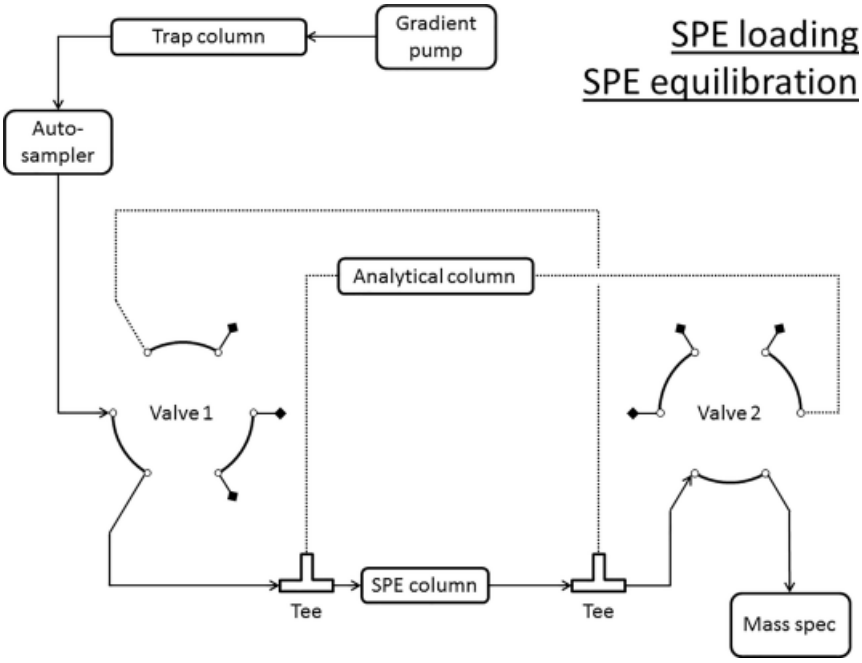

Figure 4a Flow chart of the online SPE HPLC system: SPE loading and equilibration.

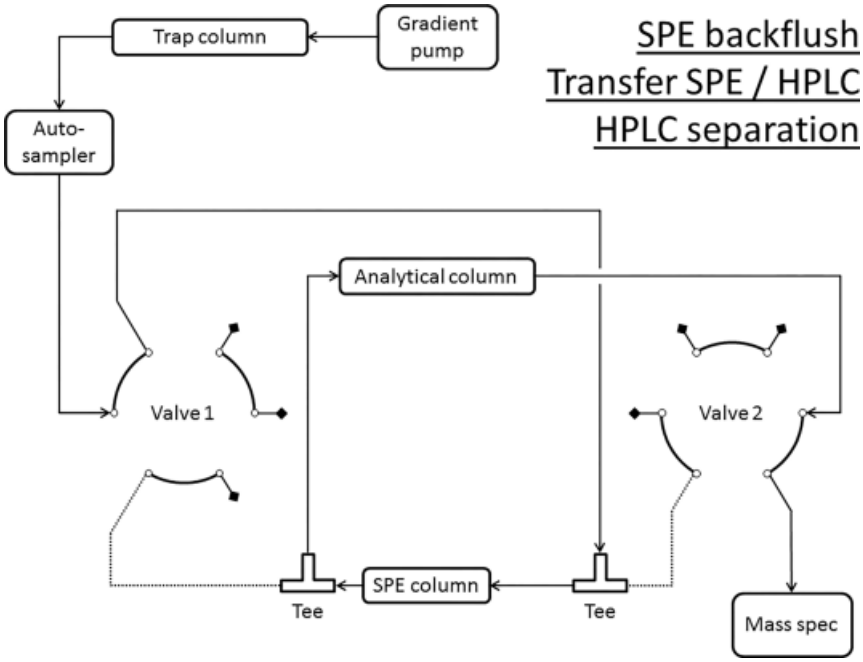

Figure 4b Flow chart of the online SPE HPLC system: SPE backflush, transfer and HPLC separation.

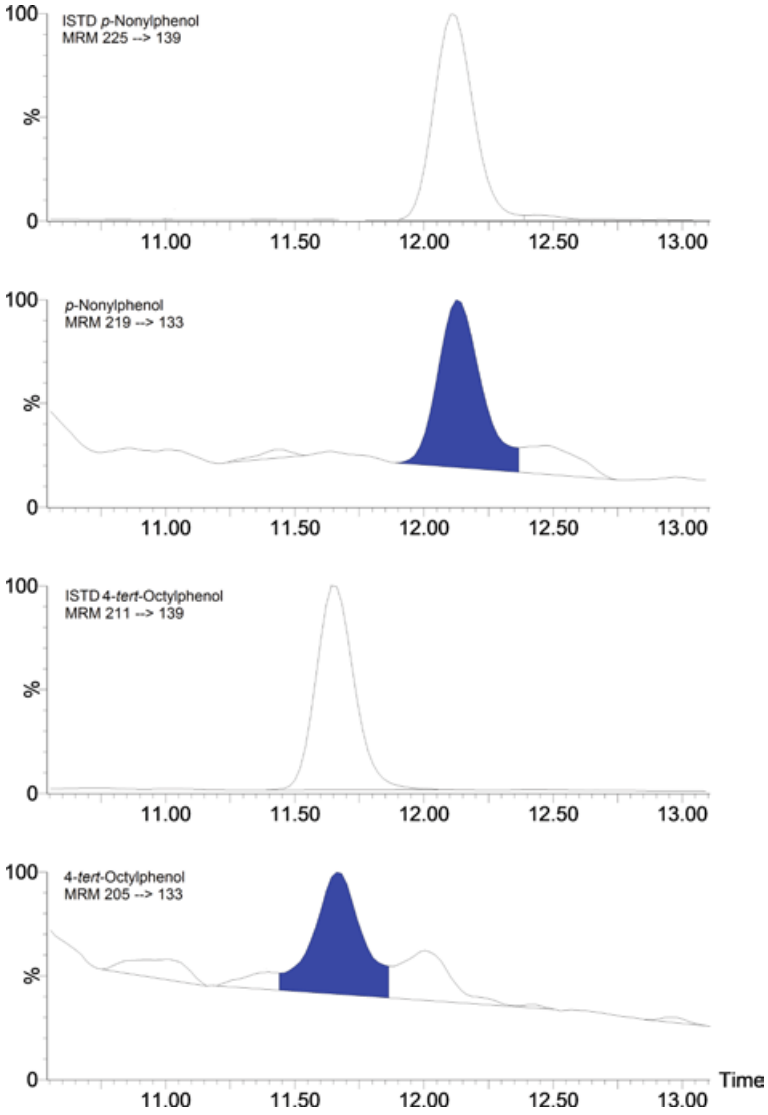

Figure 5 LC-MS/MS chromatogram of a urine sample spiked with 1 µg/L of each of the analytes.

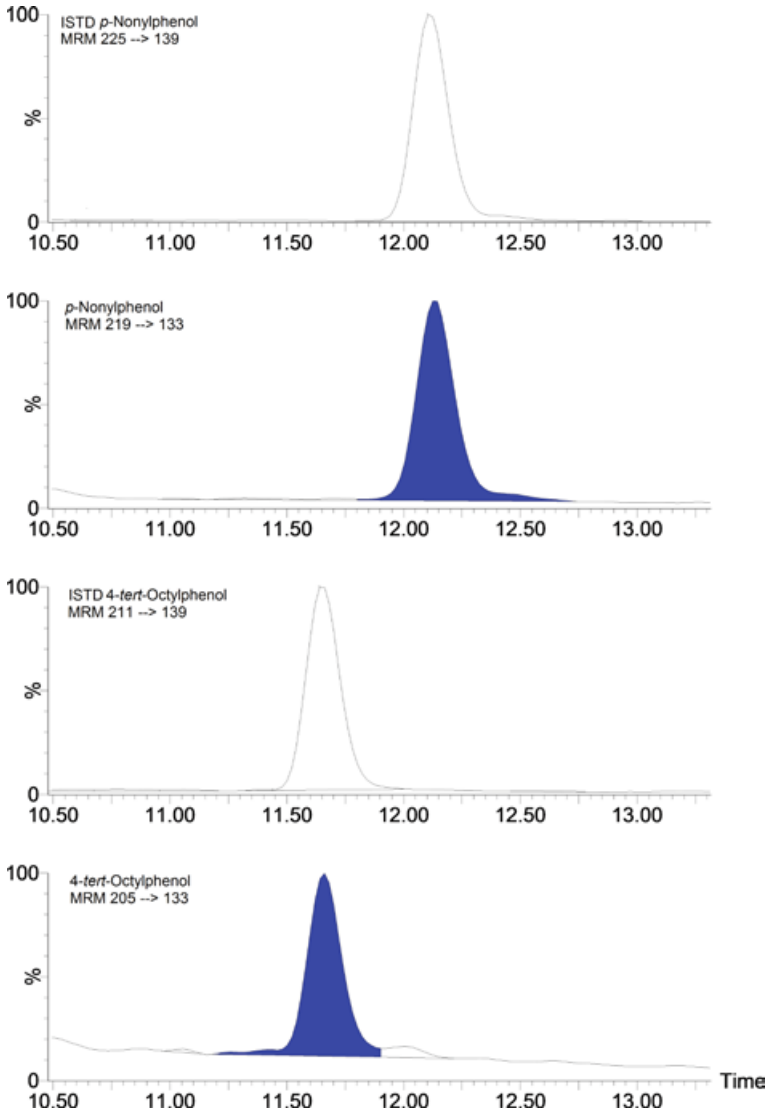

Figure 6 LC-MS/MS chromatogram of a urine sample spiked with 10 µg/L of each of the analytes.

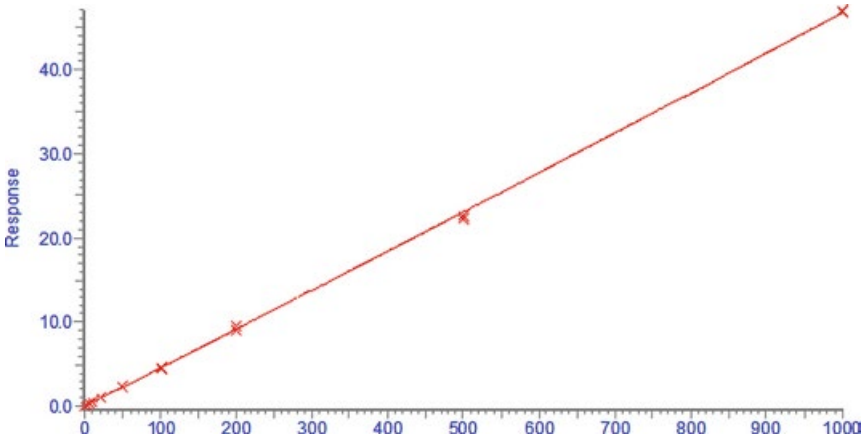

Figure 7 Calibration curve for 4-*tert*-octylphenol in urine (range 0–1000 µg/L).

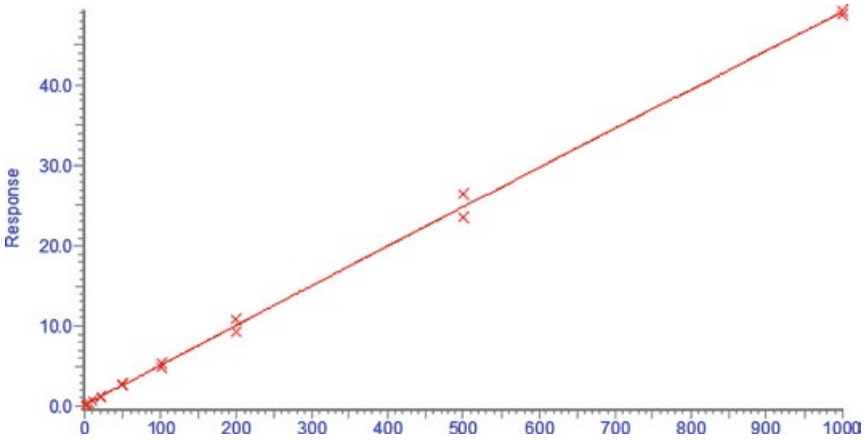

Figure 8 Calibration curve for branched *p*-nonylphenol in urine (range 0–1000 µg/L).