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Determination of chlorophenols in urine of children and suggestion of reference values

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Abstract During the course of a human biomonitoring project (Biebesheim in Hessen, Germany) we elaborated a simple but sensitive method for the determination of tri- (TCP), tetra- (TeCP) and pentachlorophenol (PCP) in human urine. Urine samples, spiked with internal standards, were treated by acid hydrolysis. After a steam bath distillation the distillates were extracted using solid phase extraction. Derivatization of the chlorophenols was not carried out. GC/ECD system was used for detection. Detection limits of the chlorophenols were found in the range of 0.02 μg/L urine (detection limits of the ECD: 0.52 to 2.76 μg/L). By this method mono- and dichlorophenols cannot be detected. We investigated 24h-urine samples of 339 pupils (age 10 to 12 years). The children live either in the surroundings of a hazardous waste incinerator (SVA) in Biebesheim (n = 193), or controls (i.e. regions without waste incinerator) in the non polluted areas of Odenwald (n = 90) and Rheintal (n = 56). Between these three groups we did not find statistically significant differences in chlorophenol concentrations of the urine samples. The 95-percentiles of the analyzed samples are 0.74 μg/L (2,3,4-TCP), 1.24 μg/L (2,3,5-TCP), 0.70 μg/L (2,3,6–TCP), 1.10 μg/L (2,4,5–TCP), 1.74 μg/L (2,4,6–TCP), 2.84 μg/L (3,4,5–TCP), 4.78 μg/L (2,3,4,5-TeCP), 1.86 μg/L (2,3,4,6-TeCP), 2.90 μg/L (2,3,5,6-TeCP) and 4.39 μg/L (PCP).

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Introduction

Chlorophenols are ubiquitously present in the environment due to a variety of industrial applications. Intake may occur dermally, orally or via the respiratory tract [1, 2]. Absorption of hexachlorocyclohexanes and chlorobenzenes leads to increased chlorophenol levels in human urine [3]. Important sources of chlorophenol exposition of the general population are food, textiles and leather goods [4][1]. Of special importance is PCP which was approved as a fungicidal wood preservative in Germany until 1989 and which was widely used both outdoors and indoors in house-building. The major portion of chlorophenols is excreted with urine, partially as free chlorophenols but mainly as sulfate or glucuronide conjugates [6]. Within recent years several authors have shown that pentachlorophenol as well as less chlorinated chlorophenol isomeres appear in urine samples of the general population [6]. These findings prompted a systematic research on the internal exposure of the general population because chlorophenols are regarded as precursors of dioxins and furans [3, 7], and PCP has proved to be carcinogenic in animal studies [8]. IARC published the results of an early study about the cancerogenic risk of PCP in 1979 [9]. Chlorophenol levels in urine samples of 339 children from Hessen were determined during the course of a human biomonitoring study. The aim of this study is to prove whether there are regional differences in contamination levels. Further, it should be proved whether chlorophenols and chlorobenzenes emitted by the hazardous waste incinerator SVA Biebesheim [10], compared to two control regions, reach the exposed population. Methods and results of this study are described.

Experimental

a) Subjects

24h-Urine samples of 339 children (age 10 to 12 years) were collected in the course of a human biomonitoring project. The chil-

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dren live in the regions Biebesheim, Rheintal and Odenwald of the Federal State Hessen (Germany). Controls are the groups Odenwald and Rheintal.

b) Reagents and solvents

Chlorophenols. Trichlorophenols (TCP): 2,3,4- (99.5%); 2,3,5- (99.0%); 2,3,6- (99.5%); 2,4,5- (99.9%); 2,4,6- (99.6%); 3,4,5- (10 ng/µL in acetonitrile); Tetrachlorophenols (TeCP): 2,3,4,5- (10 ng/µL in acetonitrile); 2,3,4,6- (97.2%); 2,3,5,6- (98.7%); Pentachlorophenol (PCP) (98.6%) (Dr. Ehrenstorfer GmbH, D-86199 Augsburg).

Internal standards: 2,6-Dibromophenol (99%; Aldrich, D-82039 Deisenhofen); 2,4,6-Tribromophenol (98.6%; Dr. Ehrenstorfer GmbH).

Solvents und reagents. Ethyl acetate (> 99.6% for residue analyses; Fluka, D-82041 Deisenhofen); Hydrochloric acid (Suprapur 30%; Merck, D-64293 Darmstadt); Sulfuric acid (Suprapur 96%; Merck); n-Undecane (≥ 99% p.a.; Merck); Ethanol (≥ 99.8% p.a.; Merck).

Solid phase extraction. Varian Bond Elut PPL columns (3 mL, 200 mg). Solid phase is a copolymer of styrene and divinylbenzene (SDB-phase) (Varian, D-64289 Darmstadt).

The columns were conditioned with 3×3 mL ethyl acetate and 2×3 mL hydrochloric acid (0.01 mol/L) and disposed after single use.

The water used for washing the SPE columns or as solvent was distilled twice with a Destamat (Heraeus Instruments GmbH, D-63405 Hanau).

c) Apparatus

GC/MS system. Finnigan MAT SSQ 7000 with Varian 3400 GC and autosampler Finnigan MAT A200S (Finnigan MAT, D-28197 Bremen); *Evaluation*: ICIS Exec 8.1.

GC system. Varian 3500 with ECD and autosampler Varian 8100; *Evaluation*: Varian Star Chromatography Workstation 5.2 software.

Gas chromatography. Capillary column: 60 m, DB-5, 0.25 mm id, 0.25 µm film thickness (J & W Scientific Products GmbH, D-50672 Köln). Injector: Split/Splitless; Split 1:30; 230 °C. Detector (ECD): 300 °C.

Temperature conditions of the column: 110 °C (5 min); 5 °C/min to 130 °C (20 min); 12 °C/min to 190 °C (10 min); 30 °C/min to 300 °C (10 min). Relays: 1; 0.60 min (split open); 30.00 min (split closed). Carrier gas: nitrogen 5.0. Inlet pressure: 20.5 psi.

SPE system. J. T. Baker SPE system with 12 connections; 75 mL reservoirs with adapters for the samples (Mallinckrodt Baker, D-64347 Griesheim).

Steam distillation. Steam generating with heated 4 liters two neck flask; steam transfer with 60 cm of a flexible teflon pipe to the sample flask; Sample is heated in a water bath (80 °C; ceran hotplate); 45 cm Liebig condenser.

d) Preparation of standard solutions in water and ethyl acetate

Internal standards in water. 25 mg of 2,6-dibromophenol and 25 mg of 2,4,6-tribromophenol were each dissolved in 25 mL ethanol (1 µg/µL; starting solutions). 1 mL of each starting solution was diluted in a 100 mL volumetric flask with bidistilled water to obtain the stock solution (10 ng/µL). Another dilution of 1 mL stock solution with bidistilled water yields the working standard (100 pg/µL).

Chlorophenol standards in water. 25 mg of the chlorophenols were each dissolved in 25 mL ethanol (1 µg/µL; starting solutions). 1 mL of each solution was diluted to a volume of 100 mL with bidistilled water (10 ng/µL; stock solutions). For the working standard 1 mL stock solution of the internal standards, 1 mL of each chlorophenol stock solution and 1 mL of 3,4,5-TCP and 2,3,4,5-TeCP, each dissolved in acetonitrile (10 ng/µL), were diluted with water in a 1000 mL volumetric flask (10 pg/µL; working solution).

Chlorophenol standards and internal standards in ethyl acetate. 10 mg of the chlorophenols, 2,6-dibromophenol and 2,4,6-tribromophenol were each dissolved in 100 mL ethyl acetate (100 ng/µL; starting solutions). 10 mL of each starting solution were diluted to 100 mL (10 ng/µL; stock solutions). 1 mL of each stock solution and 1 mL of 3,4,5-TCP and 2,3,4,5-TeCP (dissolved in acetonitrile; 10 ng/µL) were diluted to a concentration of 100 pg/µL. 40 mL of this solution were diluted with ethyl acetate in a 100 mL volumetric flask to the final concentration of 40 pg/µL (working solution).

e) Analytical procedure

Immediately after delivery of the urine samples 1 mL of acetic acid (99%) per 100 mL urine was added to each sample for preservation. The urine samples were stored in PE bottles (100 mL) at +4 °C.

One day before preparation the urine samples were warmed up to room temperature. A sample series for analysis contains a blank (20 mL of bidistilled water), an aqueous standard (20 mL of bidistilled water and 4 mL of the chlorophenol working solution) and 9 urine samples (20 mL urine).

To all samples, except the aqueous standard, 7 mL sulfuric acid (96%) and 500 µL of the aqueous working solution of the internal standards were added to 500 mL round-bottom flasks. 11 volumetric flasks (100 mL) were prepared to collect the distillates of the samples. Each flask contains 1 mL hydrochloric acid (25%).

12 hours after addition of the sulfuric acid the samples were steam distilled. Before steam distillation of every sample the apparatus was cleaned 15 min with steam by interrupting the cooling water and connecting an empty flask to the apparatus instead of the sample flask. First the blank was distilled, then the aqueous standard and the 9 urine samples. Within about 30 min 99 mL distillate of each sample were collected in the prepared flasks.

The distillates were added from a 75 mL reservoir and passed through the SPE columns within 50 to 60 min under atmospheric pressure. The solid phase was washed twice with 2 mL hydrochloric acid (0.01 mol/L) and sucked dry with air within 15 to 20 min.

The chlorophenols were desorbed with 2 mL ethyl acetate at atmospheric pressure. For that purpose the solvent was equilibrated with the solid phase for 5 min before continuing the elution. 50 µL of n-undecane were added as keeper to the collected eluates. Afterwards the eluates were evaporated under a gentle nitrogen stream to a volume of 500 µL. The eluate of the aqueous standard was evaporated to a volume of 1 mL. 1.5 µL of these samples were injected into the GC system.

f) Quality assurance and contamination control

By regular participation in external quality analyses the results of our PCP analyses (original samples) in urine were validated. Our results lay in the range of EV ± 3 × S (EV = expected value; S = standard deviation) [11].

Within each series of 9 urine samples one blank was analyzed. Insert and septum of the GC were replaced after five series each containing 9 samples, one blank and one standard. At the same time the steam bath apparatus was thoroughly cleaned.

To prove repeatability a pooled urine was analyzed 6 times. The chlorophenol concentrations found and their standard deviations are given in Table 1.

Table 1 Repeatability: means and standard deviations of chlorophenol concentrations in a pooled urine sample (analyses repeated 6 times)

Chlorophenol	Mean (µg/L)	Standard deviation (absolute, µg/L)	Standard deviation (relative, %)
2,3,4-TCP	0.217	0.007	3.0
2,3,5-TCP	0.546	0.046	8.4
2,3,6-TCP	0.216	0.018	8.4
2,4,5-TCP	0.595	0.128	21.5
2,4,6-TCP	0.612	0.043	7.1
3,4,5-TCP	0.942	0.143	15.2
2,3,4,5-TeCP	1.417	0.144	10.1
2,3,4,6-TeCP	0.859	0.060	7.0
2,3,5,6-TeCP	0.922	0.052	5.7
PCP	1.762	0.074	4.2

g) Calibration

In order to determine linearity and detection limit of the detector (ECD) calibration curves have been established for the chlorophenol concentrations ranging from 0.1 to 100 µg/L.

Table 2 shows the calculated regression coefficients of the calibration curves and the detection limits of the detector derived from their axis segments by evaluating the concentration of the standards at 2000 counts (mV · s) which is equivalent to a signal-to-noise ratio of 3:1. The detection limits of the ECD are compared to the observed detection limits of the analytical method. Because our method provides a concentration factor of 40 the ECD detection limits were divided by this factor, also shown in Table 2.

Results

Figure 1 shows the chromatograms of the standard, the blank and of a urine sample. Under the given conditions, the retention times of the chlorophenols are between 24 and 48 min. In the first chromatogram (standard) the trichlorophenols are separated properly whereas it is difficult to separate the tetrachlorophenols. The lower sensitivity of the detector to 3,4,5-TCP is conspicuous. The second chromatogram shows the result of a blank prepared with internal standards. Interfering peaks only occur in areas without influence on evaluation. The third chromatogram shows a randomly selected urine sample.

Figure 2 shows the chlorophenol concentrations in the 339 urine samples. The values for the median, 95-percentile and maximum concentration of all chlorophenols as well as the sample size are given. For 2,3,4-TCP and 2,4,5-TCP the sample sizes are reduced due to the generally low contamination and inability to identify peaks due to interfering influences.

Figure 3 shows the distribution of each chlorophenol group. The sums of the tri- and tetrachlorophenols as well as PCP are displayed. Medians and 95-percentiles are noted in the figure. The sample size rises due to the formation of sums in comparison to Fig. 2, except for PCP (n_{PCP} = 334) to n = 339. It was verified whether the distribution was influenced if samples were discarded during the formation of sums which had at least one unidentifiable chlorophenol per group. No significant influence of median or 95-percentile was found.

Discussion

In previous papers on chlorophenols in urine usually acetic anhydride [12–15] and diazomethane [16–20] were used for derivatization. Further reagents for derivatization were reviewed by Tesarova and Pacakova [21]. Generally, a GC/MS system is used for detection. We developed a simple, fast and less expensive analytical method which also ensures identification and quantification of chlorophenols in urine by GC/ECD. Using acetic anhydride and diazomethane for derivatization we found overlapping peaks in the chromatograms, therefore we discarded derivatization. Quantification is done by GC/ECD, the results were validated by GC/MS analyses of pooled urine samples. Results were verified using the retention times of the chlorophenols in combination with the selected ion monitoring spectra (selected ions: TCP: m/z 196, TeCP: m/z 232, PCP: m/z 266, 2,6-DBP: m/z 252, 2,4,6-TBP: m/z 330).

A disadvantage of our method is the fact that mono- and dichlorophenols are not quantifiable because the ECD is less sensitive to these compounds by a factor of 100 compared to higher chlorinated phenols.

Steam distillation is a method often used to separate phenolic substances from the complex urine matrix [6,

Table 2 Detection limits of the detector (ECD) and the analytical method for the determination of chlorophenols in urine (signal-to-noise ratio of 3:1)

Chlorophenol	Regression coeff. **r** of the calibration curves	Detection limits detector (µg/L) [calculated]	Detection limits detector / 40 (µg/L) [calculated]	Detection limits for urine (µg/L) [observed]
2,3,4-TCP	0.998	0.92	0.023	0.014
2,3,5-TCP	0.999	0.86	0.021	0.015
2,3,6-TCP	0.999	0.52	0.013	0.013
2,4,5-TCP	0.999	0.81	0.020	0.018
2,4,6-TCP	0.999	0.76	0.019	0.015
3,4,5-TCP	0.999	2.76	0.069	0.029
2,3,4,5-TeCP	0.999	1.15	0.029	0.015
2,3,4,6-TeCP	0.999	0.84	0.021	0.012
2,3,5,6-TeCP	0.999	1.66	0.041	0.019
PCP				0.020

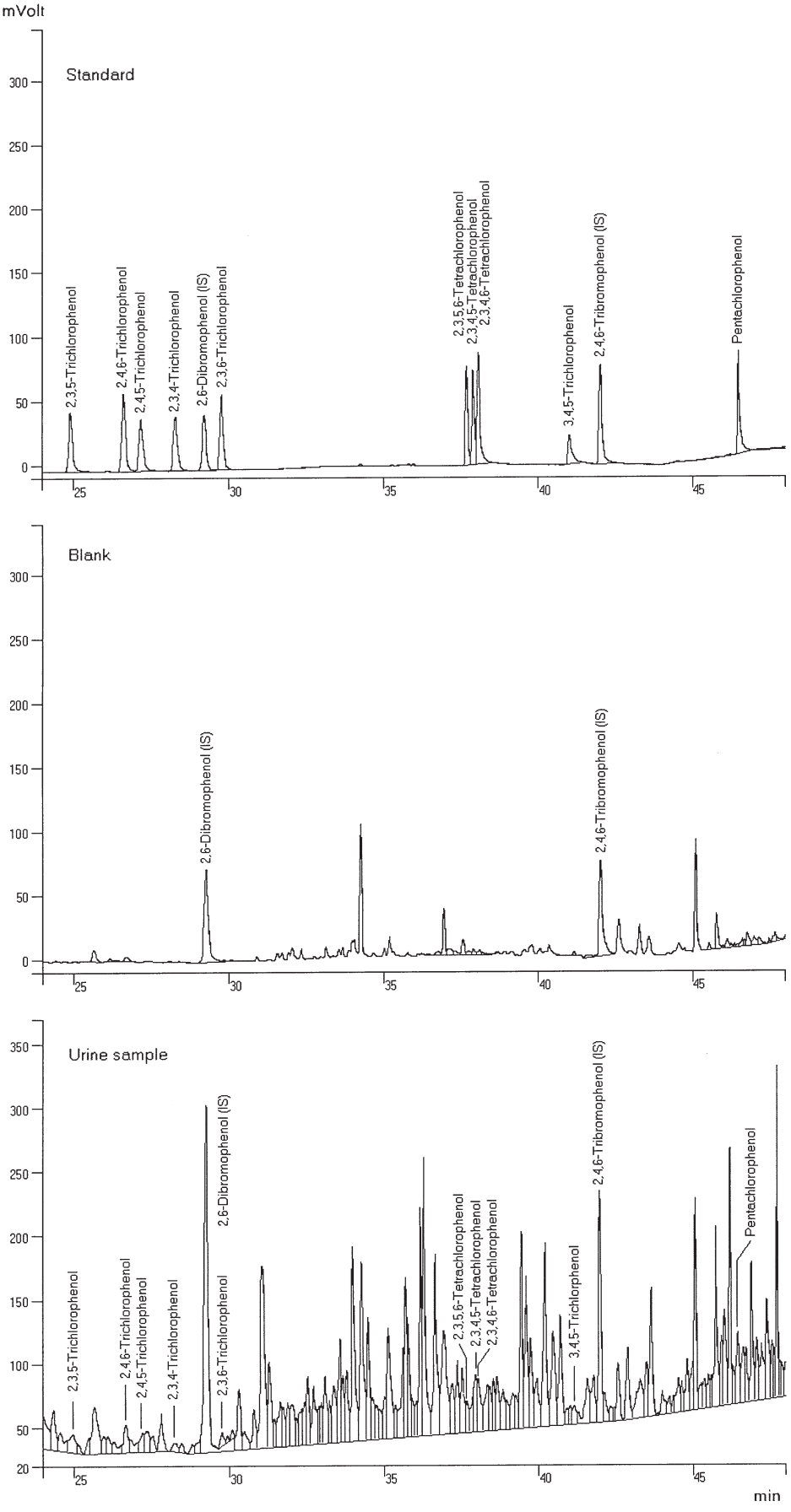

Fig. 1 Chromatograms of a working standard, a blank and a urine sample

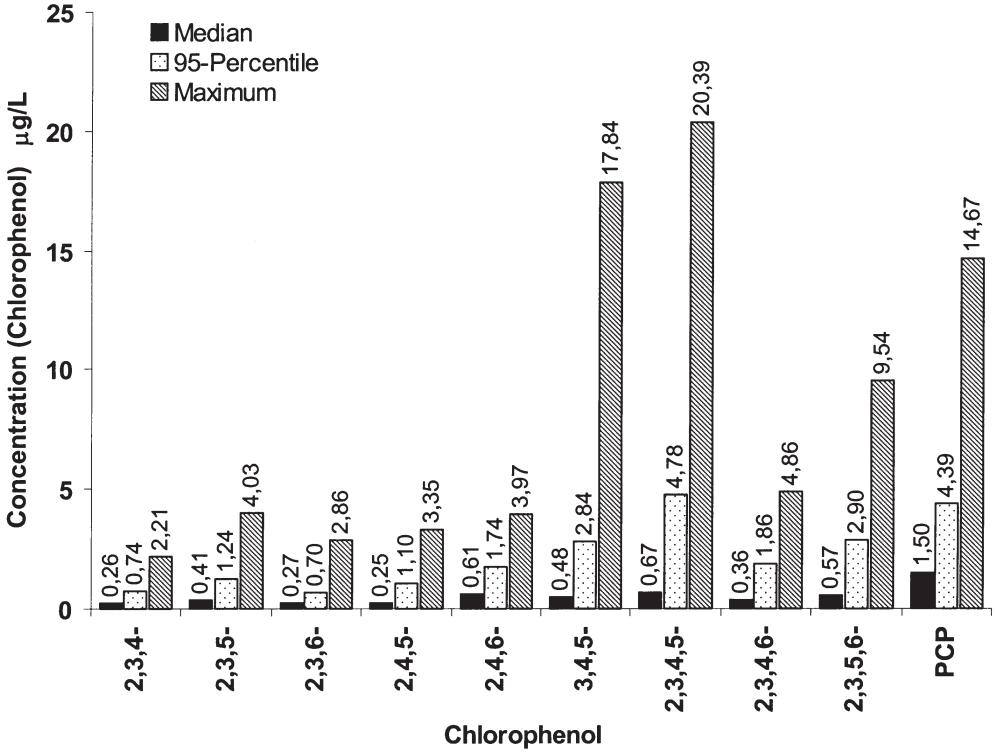

Fig. 2 Medians, 95-percentiles and maxima of chlorophenol concentrations in the urine samples of the investigated children

$n_{2,3,4}$=226	$n_{2,3,5}$=325	$n_{2,3,6}$=329	$n_{2,4,5}$=289	$n_{2,4,6}$=335
$n_{3,4,5}$=324	$n_{2,3,4,5}$=309	$n_{2,3,4,6}$=326	$n_{2,3,5,6}$=327	n_{PCP}=334

(n = number of samples)

18–22]. Two factors, however, play a decisive role. Weber [22] found that sulfuric acid in contrast to hydrochloric acid or perchloric acid provides the highest recovery for acid hydrolysis. Therefore we also decided to use sulfuric acid. Phenolic substances commonly exist in urine as sulfates or glucuronides. Pierce and Nerland [23] only found sufficient yield of phenols if higher temperature is used for the acid hydrolysis. A 90% hydrolysis of phenylglucuronide with sulfuric acid (2.5 mol/L) requires 40 min.

We optimized hydrolysis under the following conditions:

- combined hydrolysis/steam distillation of at least 30 min
- water bath temperature for the urine sample in the round-bottom flask of at least 80 °C
- a length of the Liebig condenser of 45 cm (a length of 25 cm reduced the recovery by about 80%)
- all-glass steam distillation apparatus in order to avoid contamination (with a flexible Teflon pipe between the steam generator and the sample flask)
- purification of the apparatus with steam (interruption of cooling water for about 15 min) between two sample distillations in order to avoid cross contamination

After a comparison of solid phase extraction (RP18 versus SDB-phases) using a sufficient eluting volume, Schilling et al. [24] found an enhanced recovery for the SDB-phases for phenolic substances. We decided to use a suitable SDB-phase, because the combination of a cation exchanger phase and a C_{18}-phase used by Angerer et al. [6] revealed no advantage.

The chlorophenols were eluted with the lowest possible volume of ethyl acetate in order to reduce the risk of losses in the following evaporation step under N_2. Before the evaporation step each eluate is prepared with a keeper (50 µL n-undecane). Our method provides a concentration factor of 40.

The chlorophenol standard in ethyl acetate (working solution) ist stable for about 3 weeks at +4 °C.

The calculation of the results is based on both the mean recovery of 2,6-dibromophenol and 2,4,6-tribromophenol, which were regularly used for the identification of chlorophenols [1, 6, 17] and on the individual recovery of the respective chlorophenol (obtained from the sample with the aqueous standard solution). Thus, the specific recovery of each chlorophenol was considered. The blank of each series is subtracted.

Concerning the detected concentrations of chlorophenols, one cannot, so far, decide whether a risk to health exists.

In 1984 a study on 27 persons without occupational and private exposure to PCP (e.g. by wood preservatives) showed a median PCP level in urine of 10 µg/L (95-per-

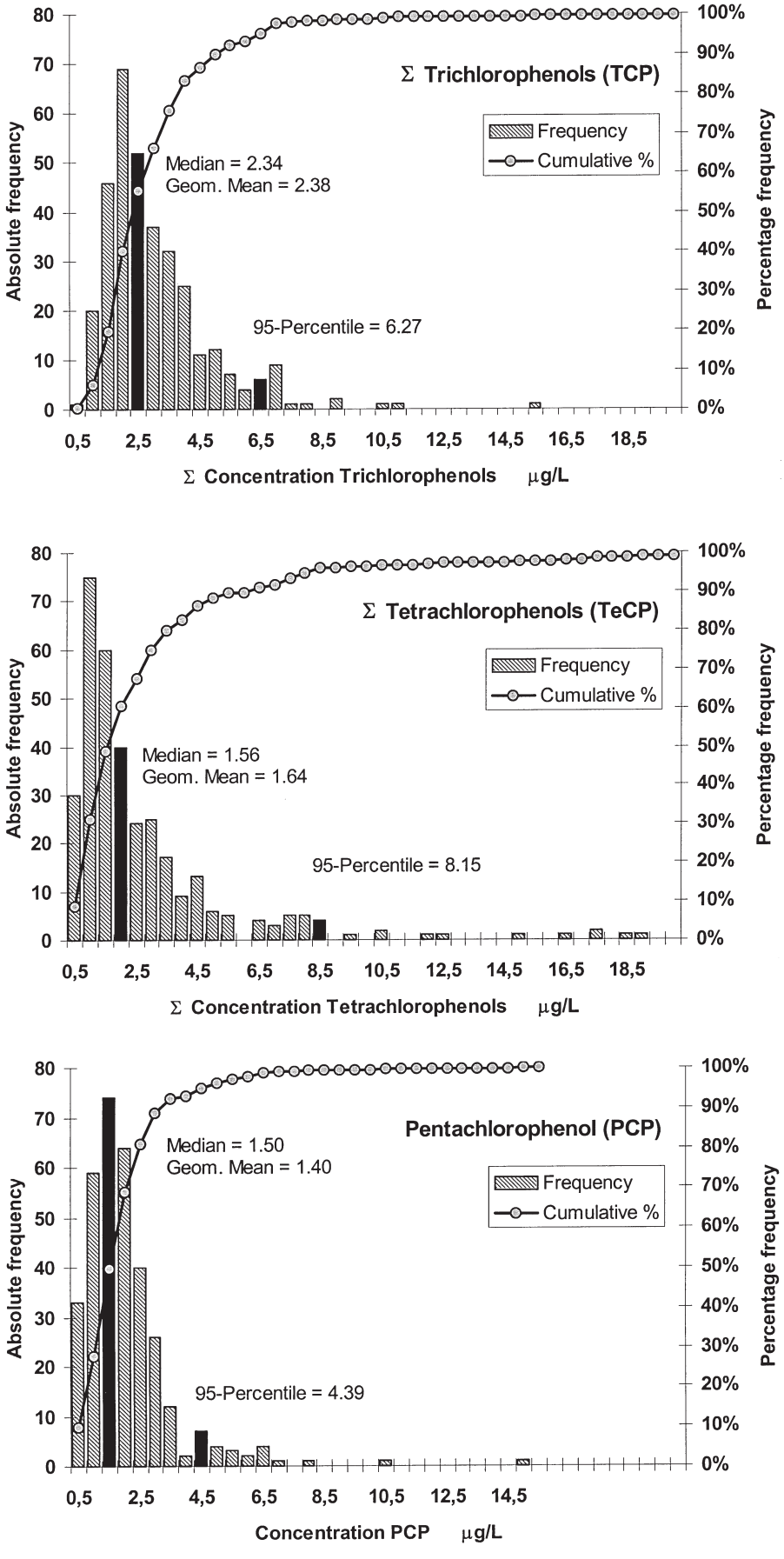

Fig. 3 Frequency distribution of the chlorophenols (n_{TCP} = 339, n_{TeCP} = 339, n_{PCP} = 334)

centile: 55 μg/L) [17]. During the last decade a significant decrease of PCP input both in human and in natural environment could be monitored in Germany. In 1995 a "reference value" for PCP (morning urine) of 15 μg/L (n = 112 individuals; age 37 to 86 years; without known exposure to wood preservatives) was published [25]. In 1997 a revised "reference value" of 4 μg/L (spontaneous urine; n = 255) was recommended [26]. Treble analyzed 1995 69 urine samples from non-occupationally exposed subjects (age 6 to 87 years; residence: various rural and urban areas throughout Saskatchewan). The PCP concentrations ranged from 0.05 to 3.6 μg/L [27].

The 95-percentile of our PCP analyses (4.4 μg/L; age 10 to 12 years) is within the same range. Besides the direct PCP uptake, especially due to its occurrence in wood, textiles and leather materials, PCP is a main metabolite of hexachlorobenzene (HCB) [3, 28]. The Umweltprobenbank in Münster correlates the concentration of PCP in urine with the concentration of HCB in serum [29].

Concerning the individual isomeres, like tri- and tetrachlorophenols the data basis for "reference values" in urine is insufficient. Our analyses revealed for 2,3,4,5-TeCP (95–percentile: 4.8 μg/L; maximum: 20.4 μg/L) the highest concentration among the detected chlorophenols. Wrbitzky et al. explain its occurrence in urine by the metabolism of 1,2,3,4-tetrachlorobenzene (e.g. from the production of fungicides and dielectrics) and γ-HCH [3]. Reported exposition to wood preservatives, HCB and other confounders (e.g. living circumstances, life style, foodstuffs from the area of the SVA Biebesheim etc.) may explain our findings. This question and the possible role of polychlorinated dibenzodioxins and furans as known impurities of technical PCP [30, 31] and other chlorophenols will be discussed in a further publication.

The aim of the investigation was to compare the population's contamination levels from different regions in Hessen. Another aim was to evaluate reference values for chlorophenols in urine. Such values allow an estimation of the extent of background contamination.

Because there are no significant regional differences in the chlorophenol contamination levels, our results can be used for the finding of reference values for the chlorophenol concentrations in urine.

The 95-percentile of the observed distribution of the measured concentrations in a reference group is used [32]. It is required that this representative reference group is not specifically contaminated. The groups of children we investigated showed no statistically significant regional differences in the contamination levels. Our reference values (95-percentiles) are shown in Figs. 2 and 3, and they facilitate a comparison of individual urine concentrations of chlorophenols with the general background contamination. Such values, however, should not be misunderstood as toxicological risk assessment [32].

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