Supporting Information

Urinary excretion of phthalate metabolites in school children of China: Implication for cumulative risk assessment of phthalate exposure

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Table S1. The demographic characteristics of participants in three sampling areas

	All (%)	Haimen[a]	Minhang	Yuhuan
Gender				
Male	407	139	136	132
Female	375	126	118	131
Age (years)	9.7 ± 0.9[b]	9.6 ± 1.0	9.6 ± 0.9	9.9 ± 0.9
8	70	31	22	17
9	277	99	100	78
10	241	74	84	83
11	194	61	48	85
Weight (kg)	34.2 ± 8.8[b]	33.0 ± 8.6	36.4 ± 9.4	33.4 ± 8.1
Height (cm)	139.9 ± 8.4[b]	138.4 ± 8.4	142.4 ± 7.3	138.9 ± 8.7
Creatinine (mg/dl)	60.4 ± 41.5 [b]	51.0 ± 36.4	55.6 ± 32.5	74.7 ± 49.5

[a] Data of weight and height was missing for one children.

[b] Mean ± Std. Deviation.

Sample pretreatment

Urine samples were analyzed for 13 phthalate metabolites: mono (2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono (2-ethyl-5-carboxypentyl) phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP), mono[(2-carboxymethyl) hexyl] phthalate (MCMHP), monomethyl phthalate (MMP), monocyclohexyl phthalate (MCHP), monobenzyl phthalate (MBzP), mono-3-methyl-5-dimethylhexyl phthalate (isononyl, MiNP) and monooctyl phthalate(MOP). Thirteen metabolites, six isotopically-labeled internal standards ($^{13}C_4$-MMP, $^{13}C_4$-MEP, $^{13}C_4$-MnBP, $^{13}C_4$-MECPP, $^{13}C_4$-MEHP, $^{13}C_4$-MBzP) and $^{13}C_4$-4- methylumbelliferone were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 4-methylumbelliferone glucuronide and β-glucuronidase from Helix pomatia were purchased from Sigma-Aldrich (St. Louis, MO, USA).

An aliquot (1.0 mL) of urine was spiked with 50 μL of stand solution containing 500 ng/mL of six mixed isotopically labeled phthalate metabolites, 500ng/mL of 13C_4-4-methylumbelliferone, and 1000 ng/mL of 4-methylumbelliferone glucuronide. After 30min of equilibration, the sample was buffered with 200 μL of 1.0 M ammonium acetate buffer (pH 5.0; 7.7 g of ammonium acetate dissolved in 100 mL of Milli-Q water and added 6 mL of acetic acid) and added by 15μL of β-glucuronidase. All the samples were then incubated at 37℃for deconjugation overnight.

After enzymatic deconjugation, the urine samples were loaded onto Oasis MAX solid-phase extraction (SPE) anion exchange cartridges (150mg/mL, Waters, Milford, MA). The cartridges were previously preconditioned with 4 mL of methanol and 4 mL of Milli-Q water. Ammonium acetate buffer was washed from the cartridges by 4 mL of pure water, and 4 ml of 50 mM sodium bicarbonate buffer (pH 8.5; 2.1 g of sodium bicarbonate dissolved in 500 mL of Milli-Q water)

was used to promote the binding of phthalate metabolites to SPE material. After neutral or basic compounds were washed from the cartridges by 4 mL of methanol, the SPE material was under vacuum for 10 min. The target phthalate metabolites were eluted by 5 mL of methanol containing 2% formic acid. Flow rate of loading, washing, and elution in SPE process was kept at 1.5 mL/min. The eluate was concentrated to dryness with a gentle stream of nitrogen in a 45℃ water bath, and reconstituted in 0.5mL of 30% acetonitrile aqueous solution.

Instrumental Analysis

An Acquity ultra-performance liquid chromatography (UPLC) coupled with a Xevo TQ triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used for the measurement of target compounds. Ten microliters of final solution were injected onto an Acquity UPLC C18 column (Acquity UPLC BEH C18, 100 mm×2.1 mm×1.7 μm). Target compounds were eluted by water (A) and acetonitrile (B) both containing 0.1% acetic acid at a flow rate of 300μL/min. The mobile phase gradient was as follows: 0-0.2min, 5%B; 0.2-1.0min, 25%B; 1.0-9.0min, 55%B; 9.0-11.0min, 100%B; 11.0-11.5min, 5%B; 11.5-14.0min, 5%B. The column temperature was set to 40℃.

Target compounds were analyzed under negative ion (NI) mode. Nitrogen was used as the desolvation gas. The desolvation gas flow was set to 800L/h and its temperature was set to 400℃. The cone gas flow was set to 40 L/h, and the source temperature was set to 120℃. The capillary voltage was set to 2.8 KV. The multiple reaction monitoring (MRM) mode was used, and the specific setting was listed in Table S2.

$^{13}C_4$-MMP was used as a surrogate standard for quantification of MMP, $^{13}C_4$-MEP for MEP, $^{13}C_4$-4-methylumbelliferone for 4-methylumbelliferone, $^{13}C_4$-MECPP for MECPP and MEHHP, $^{13}C_4$-MnBP for MnBP, MiBP, MEOHP, and MCMHP, $^{13}C_4$-MBzP for MBzP and MCHP, $^{13}C_4$-MEHP for MEHP, MOP, and MiNP.

MEHP, MiBP, MnBP, MMP, and MEP were detected in procedural blanks, and the average

blank values for these metabolites were 0.91, 1.22, 1.51, 0.63, 1.18, respectively. The final study sample concentrations for these compounds were determined as their initial measurements minus the concentrations found in the blanks. LOD was calculated as a signal−to−noise ratio of 3, from the chromatograms of urine samples spiked with the lowest concentration of analyte tested.

Table S2. Settings for multiple reaction monitoring (MRM) mode

Compounds	Retention time (min)	Cone voltage (V)	Collision energy (V)	Precursor ion[a]	Product ion
MEHP	10.81	25	19	277	134, 233,147
MECPP	5.58	15	20	307	159, 121
MEHHP	5.92	25	26	293	121, 145
MEOHP	6.08	25	21	291	121, 143
MCMHP	6.29	10	20	307	159, 113
MiBP	6.01	20	24	221	177, 121, 147
MBP	6.15	20	21	221	177, 147, 134
MMP	3.05	15	23	179	77, 107, 121
MEP	3.74	20	25	193	77, 121, 147
MCHP	7.22	25	25	247	77, 121, 147
MBzP	6.44	20	17	255	183,147. 105
MiNP	11.06	30	20	291	141, 219, 247
MOP	11.04	25	22	277	127, 233, 205

[a][M-H]$^-$ for phthalate metabolites.

Table S3. The Results of Quality Assurance/Quality Control

Compounds	Linear regression coefficient (r)	Recovery (%)	RSD (%)	LOD (ng/mL)
MEHP	0.998	84.2-124	19.1	1.03
MECPP	0.996	82.6-113	15.5	0.56
MEHHP	0.996	86.5-108	11.1	0.39
MEOHP	0.996	76.8-92.3	9.2	0.35
MCMHP	0.996	80.6-103	12.2	0.21
MiBP	0.999	72.2-94.3	13.3	1.48
MnBP	0.996	92.3-117	11.8	1.81
MMP	0.996	76.4-98.6	12.7	0.87
MEP	0.998	70.3-98.0	16.5	1.62
MCHP	0.998	86.9-118	15.2	0.18
MBzP	0.998	83.2-106	12.1	0.14
MiNP	0.998	95.6-121	11.7	0.11
MOP	0.999	88.6-117	13.8	0.11

Table S4. Data used for the estimation of the target phthalates daily intake

Phthalate	Molecular weights	Phthalate metabolite	Molecular weights	Excretion fraction		Reference
Di-n-butyl phthtalate(DnBP)	278	Mono-n-butyl phthalate(MnBP)	222	0.69		Anderson et al.(2001)
Di-isobutyl phthalate(DiBP)	278	Mono-isobutyl phthalate(MiBP)	222	0.703		Koch et al.(2012)
Diethyl phthalate(DEP)	222	Monoethyl phthalate(MEP)	194	0.69[a]		Anderson et al.(2001)
Dimethyl phthalate(DMP)	194	Monomethyl phthalate(MMP)	180	0.69		Itoh et al.(2007)
Butylbenzyl phthalate(BBzP)	312	Monobenzyl phthalate(MBzP)	256	0.73		Anderson et al.(2001)
Di(2-ethylhexyl)phthalate(DEHP)	390	Mono (2-ethylhexyl) phthalate(MEHP)	278	0.062	Sum:0.453	Anderson et al.(2011)
		Mono(2-ethyl-5-oxohexyl)phthalate(MEOHP)	292	0.109		
		Mono(2-ethyl-5-carboxypentyl)phthalate(MECPP)	308	0.132		
		Mono(2-ethyl-5-hydroxyhexyl)phthalate(MEHHP)	294	0.149		

[a]No excretion factor available; factor determined by analogy to MnBP(Koch et al., 2009).

Table S5. Urinary phthalate metabolite concentrations (median in ng/mL) in China, compared with other human biomonitoring studies on children

Region/Reference	Sampling year	Age(n)	Phthalate metabolites											
			MMP	MiBP	MnBP	MEP	MCHP	MBzP	MEHP	MEOHP	MEHHP	MECPP	MCMHP	$\sum DEHP_m$
USA														
CDC (2014)	2009-2010	6-11y(415)	2.36	10.9	23.3	33.0	<LOD[a]	12.6	1.71	11.1	17.0	29.4	-	-
Germany														
Becker et al.(2009)	2003-2006	3-14y(599)	-	88.1	93.4	-	-	18.1	6.7	36.3	46.0	61.4	20.4	-
		9-11y(149)	-	91.2	92.2	-	-	16.0	6.8	38.5	45.9	58.2	20.3	-
Koch et al.(2011)	2007	5-6y(111)	-	42.8	36.8	-	-	7.2	4.7	15.1	17.4	28.4	11.3	-
Kasper-Sonnenberg et al.(2012)	2007-2009	6-8y(234)	3.8	68.7	54.2	33.6	<LOQ[b]	11.7	4.0	26.4	31.0	42.1	-	57.2
Denmark														
Frederiksen et al.(2013)	2011	6-11y(143)	-	54.0	32.0	20.0	-	7.0	2.0	12.0	23.0	15.0	-	69.0
Frederiksen et al.(2014)	2006-2008	5-9y(556)	-	80.6	55.6	33.6	-	48.2	4.63	26.4	50.3	30.1	-	145
		10-13y(538)	-	76.5	47.3	38.7	-	50.7	5.22	24.2	47.5	27.4	-	140
Canada														
Saravanabhavan et al. (2013)	2007-2009	6-11y(1037)	<LOD[c]	-	32.6	23.6	<LOD[d]	21.4	3.3	20.3	31.6	-	-	-
China														
This study	2012	8-11y(782)	8.4	38.5	47.1	18.7	0.8	0.3	5.9	17.6	11.8	20.7	11.8	64.7

[a] LOD=0.402 ng/mL.

[b] LOQ=0.5 ng/mL.

[c] LOD=5 ng/mL.

[d] LOD=0.2 ng/mL.

Table S6. Spearman correlations of urinary phthalate metabolites concentrations (uncorrected)

	MMP	MiBP	MnBP	MEP	MCHP	MBzP	MEHP	MEOHP	MEHHP	MECPP	MCMHP
MMP	1	**0.65****	**0.53****	**0.59****	0.27**	- 0.1**	0.35**	**0.57****	**0.53****	**0.59****	**0.61****
MiBP		1	0.41**	**0.56****	0.33**	- 0.07	0.29**	**0.57****	**0.52****	**0.58****	**0.6****
MnBP			1	0.45**	0.26**	- 0.03	0.27**	**0.51****	0.44**	**0.51****	**0.51****
MEP				1	0.29**	- 0.11**	0.27**	0.45**	0.4**	0.45**	0.48**
MCHP					1	- 0.09*	0.25**	0.25**	0.31**	0.23**	0.24**
MBzP						1	0.01	- 0.01	- 0.04	- 0.01	- 0.03
MEHP							1	**0.66****	**0.63****	**0.63****	**0.6****
MEOHP								1	**0.87****	**0.95****	**0.94****
MEHHP									1	**0.84****	**0.82****
MECPP										1	**0.95****
MCMHP											1

** $p<0.01$; * $p<0.05$.

Table S7. Median (interquartile range) concentrations of phthalate metabolites (in ng/mL and in μg/g creatinine) in urine samples of Chinese children in relation to gender and age

	Gender			Age (years)		
	Male (n=407)	Female (n=375)	*P*-value	8-9 (n=347)	10-11 (n=435)	*P*-value
MMP	8.5(13.9)	8.3(12.5)		7.4(13.7)	8.8(12.3)	
	15.6(18.7)[a]	**15.0(20.0)**	0.956	**15.7(21.0)**	**15.3(18.3)**	0.746
MiBP	44.7(62.0)	32.9(46.1)		36.4(52.8)	40.4(53.0)	
	85.4(96.0)	**66.0(75.0)**	**0.004**	**71.5(93.2)**	**77.0(82.0)**	0.774
MnBP	46.7(73.5)	47.6(95.9)		40.7(77.2)	52.0(86.6)	
	92.5(152)	**94.7(148)**	0.824	**96.2(148)**	**90.4(155)**	0.94
MEP	19.4(54.7)	18.0(50.3)		17.5(40.8)	19.5(66.0)	
	38.7(87.6)	**41.0(88.6)**	0.32	**40.5(85.8)**	**40.4(101)**	0.321
MCHP	0.8(1.0)	0.8(0.9)		0.6(0.9)	0.9(1.0)	
	1.5(1.9)	**1.6(2.2)**	0.567	**1.5(2.1)**	**1.5(2.1)**	0.534
MBzP	0.7(0.1)	0.3(0)		0.3(0)	0.3(0.1)	
	0.6(0.7)	**0.6(0.7)**	0.55	**0.6(0.7)**	**0.5(0.6)**	**0.004**
MEHP	6.2(6.6)	5.7(5.6)		5.7(5.8)	6.0(6.1)	
	12.0(15.6)	**11.5(14.6)**	0.505	**12.5(15.3)**	**10.6(15.2)**	0.06
MEOHP	18.6(20.7)	16.6(21.7)		18.5(22.6)	16.6(20.4)	
	38.2(37.7)	**33.8(35.1)**	0.173	**40.7(36.7)**	**30.6(33.4)**	**＜0.001**
MEHHP	12.0(17.8)	11.6(14.6)		13.3(17.6)	10.5(15.0)	
	23.8(33.3)	**23.3(28.4)**	0.981	**30.0(35.8)**	**20.2(25.6)**	**＜0.001**
MECPP	22.0(26.8)	19.5(29.4)		22.4(29.1)	19.5(27.6)	
	40.4(46.2)	**40.0(45.1)**	0.372	**44.8(48.6)**	**35.2(39.7)**	**＜0.001**
MCMHP	12.6(16.7)	11.0(15.7)		12.2(18.1)	11.5(16.1)	
	23.5(25.2)	**22.7(25.8)**	0.236	**26.7(26.3)**	**20.6(24.2)**	**＜0.001**
$\sum DEHP_m$[b]	68.0(75.2)	62.1(81.3)		66.2(86.9)	62.9(77.1)	
	137 (140)	**128 (139)**	0.447	**147 (139)**	**112 (125)**	**＜0.001**

[a] Bold type: creatinine corrected concentration (in μg/g creatinine).

[b] $\sum DEHP_m$ is the sum of 5 DEHP metabolites.

Table S8. Significance demonstration (p values) for the differences of phthalate metabolite concentrations (in creatinine-adjusted) between three sampling areas[a]

	Haimen-Minhang-Yuhuan	Haimen-Minhang	Haimen-Yuhuan	Minhang-Yuhuan
MMP	**<0.001**	**<0.001**	**<0.001**	**<0.001**
MiBP	**<0.001**	**0.003**	**<0.001**	**<0.001**
MnBP	**<0.001**	**<0.001**	**0.009**	**<0.001**
MEP	**<0.001**	**0.002**	**<0.001**	**<0.001**
MCHP	**<0.001**	**<0.001**	0.089	**<0.001**
MBzP	**<0.001**	0.998	**<0.001**	**<0.001**
MEHP	**<0.001**	**<0.001**	**<0.001**	**0.001**
MEOHP	**<0.001**	**<0.001**	**<0.001**	0.04
MEHHP	**<0.001**	**<0.001**	**0.001**	**<0.001**
MECPP	**<0.001**	**<0.001**	**<0.001**	0.181
MCMHP	**<0.001**	**<0.001**	**<0.001**	**0.014**
$\sum DEHP_m$	**<0.001**	**<0.001**	**<0.001**	**0.006**

[a] Statistical significance was set at Bonferroni-adjusted p-value.

Table S9. Estimated daily intake of phthalates (in μg/kg body weight/day) for Chinese children, compared to daily intakes for children from other studies

Region/Reference	Sampling year	Age(n)	DiBP		DnBP		DEP		BBzP		DEHP	
			Median	P95	Median	P95	Median	P95	Median	P95	Median	P95
Creatinine-based model												
USA												
Christensen et al.(2014)[a]	2005-2008	6-11y(742)	0.4	1.49	0.9	3.5	-	-	0.7	3.4	6.0	40.9
Denmark												
Frederiksen et al.(2013)	2011	6-11y(141)	2.4(2.8)[b]	7.6(7.4)	0.7(0.9)	2.2(2.0)	0.5(0.5)	3.0(2.7)	0.2(0.2)	1.1(1.1)	2.7(2.4)	8.1(12.5)
Germany												
Wittassek et al.(2007), Koch et al.(2007)	2001-2002	2-14y(239)	-	-	4.1	14.9	-	-	0.4	2.6	4.3	15.2
Koch et al.(2011)	2007	5-6y(108)	2.1	11	1.9	6.4	-	-	0.3	2.6	4.5	18
China												
This study	2012	8-11y(782)	2.6	11.8	3.3	20.0	1.2	18.2	0.02	0.1	6.5	27.1
Volume-based model												
Germany												
Wittassek et al.(2007), Koch et al.(2007)	2001-2002	2-14y(239)	-	-	7.6	30.5	-	-	0.8	4.5	7.8	25.2
Denmark												
Frederiksen et al.(2011)	2007	6-21y(129)	-	-	4.3[c]	11.3[c]	1.1	8	0.6	3.8	4.0	10.7
Bekö et al.(2013)	2008-2009	3-6y(431)	2.9	10	3.3	10	0.6	3.9	0.5	2.8	4.4	16.9
Belgium												
Dewalque et al.(2014)	2013	1-12y(52)	2.3	8.0	2.4	7.3	1.5	5.8	0.4	1.7	3.4	10.6
China												
This study	2012	8-11y(782)	1.5	7.6	1.9	14.2	0.7	13.2	0.01	0.04	3.7	18.3

[a] Estimated using biomonitoring data from NHANES 2005-2006 and 2007-2008.

[b] Estimated daily intake for children from urban area(outside the bracket) and from rural area(inside the bracket).

[c] MnBP and MiBP analyzed together as one.

Table S10. Estimated daily intake of phthalates (in μg/kg body weight/day) between selected demographic groups

	N(vol)/ N(cre)	Median (95th percentile)											
		DMP		DiBP		DnBP		DEP		BBzP		DEHP	
		Vol-model	Cre-model	Vol-model	Cre-model	Vol-model	Cre-model	Vol-model	Cre-model	Vol-model	Cre-model	Vol-model	Cre-model
Gender													
Male	407/407	0.3(2.1)	0.5(3.5)	1.8(8.3)	2.9(12.2)	1.9(14.2)	3.2(19.8)	0.7(11.2)	1.2(16.8)	0.01(0.05)	0.017(0.125)	3.9(21.1)	6.7(29.6)
Female	375/374	0.3(1.6)	0.5(2.5)	1.3(6.9)	2.3(11.0)	1.9(14.4)	3.3(20.7)	0.7(15.7)	1.3(34.7)	0.01(0.02)	0.019(0.083)	3.5(14.7)	6.4(22.9)
p-value		0.634	0.697	**0.004**	**0.014**	0.648	0.962	0.801	0.316	0.199	0.88	0.143	0.633
Age(years)													
8-9	347/347	0.3(1.7)	0.5(3.3)	1.5(7.4)	2.5(12.6)	1.7(10.6)	3.3(17.3)	0.65(8.9)	1.2(17.1)	0.01(0.04)	0.020(0.119)	3.8(16.9)	7.4(28.0)
10-11	435/434	0.3(1.9)	0.5(2.8)	1.6(9.0)	2.7(11.7)	2.1(15.7)	3.3(22.1)	0.72(15.9)	1.3(26.8)	0.01(0.04)	0.016(0.097)	3.5(18.9)	5.7(26.7)
p-value		0.119	0.776	**0.029**	0.667	**0.039**	0.963	**0.018**	0.264	0.725	**0.006**	0.175	**＜0.001**
Region													
Haimen	265/264	0.3(1.3)	0.5(3.2)	1.3(5.1)	2.4(11.7)	1.9(9.5)	3.4(22.3)	0.5(9.9)	1.1(20.9)	0.01(0.03)	0.021(0.116)	4.4(20.9)	8.8(40.8)
Minhang	254/254	0.2(0.8)	0.3(1.2)	1.1(3.8)	1.9(7.2)	1.2(6.2)	2.3(12.1)	0.4(3.7)	0.8(6.4)	0.01(0.04)	0.020(0.102)	2.8(13.0)	5.1(17.7)
Yuhuan	263/263	0.5(4.2)	0.7(7.7)	2.6(14.6)	3.6(14.8)	3.0(18.8)	4.3(22.1)	1.8(36.9)	2.3(44.8)	0.01(0.04)	0.014(0.082)	3.9(20.6)	6.5(18.6)
p-value		**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**	**＜0.001**

Vol-model: volume-based model; Cre-model: creatinine-based model.

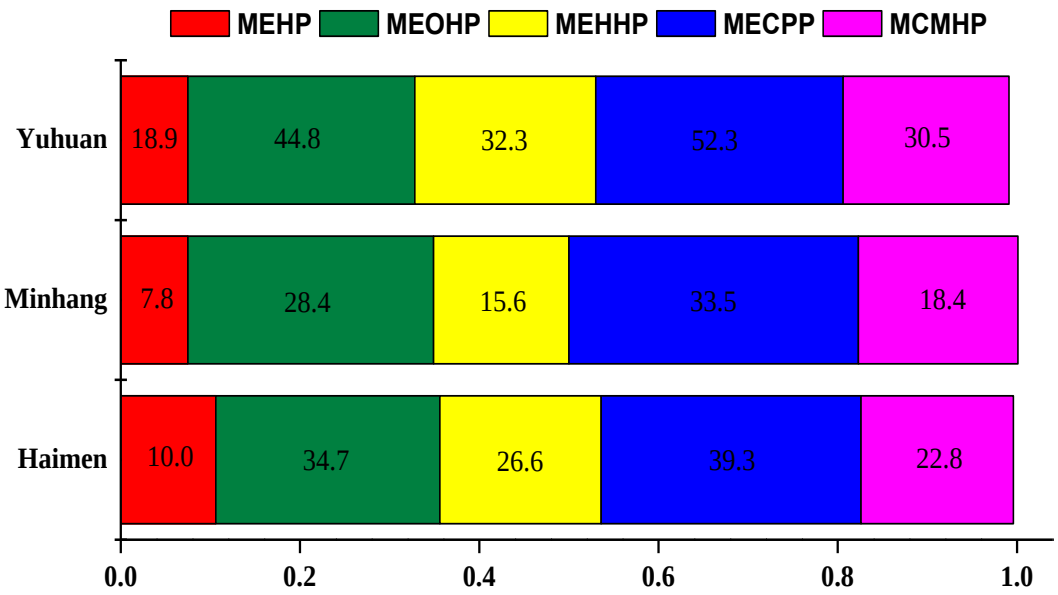

Figure S1. Compositions of DEHP metabolites in urine samples from three regions in China. The figures in the bars present median concentrations (in μg/g creatinine)

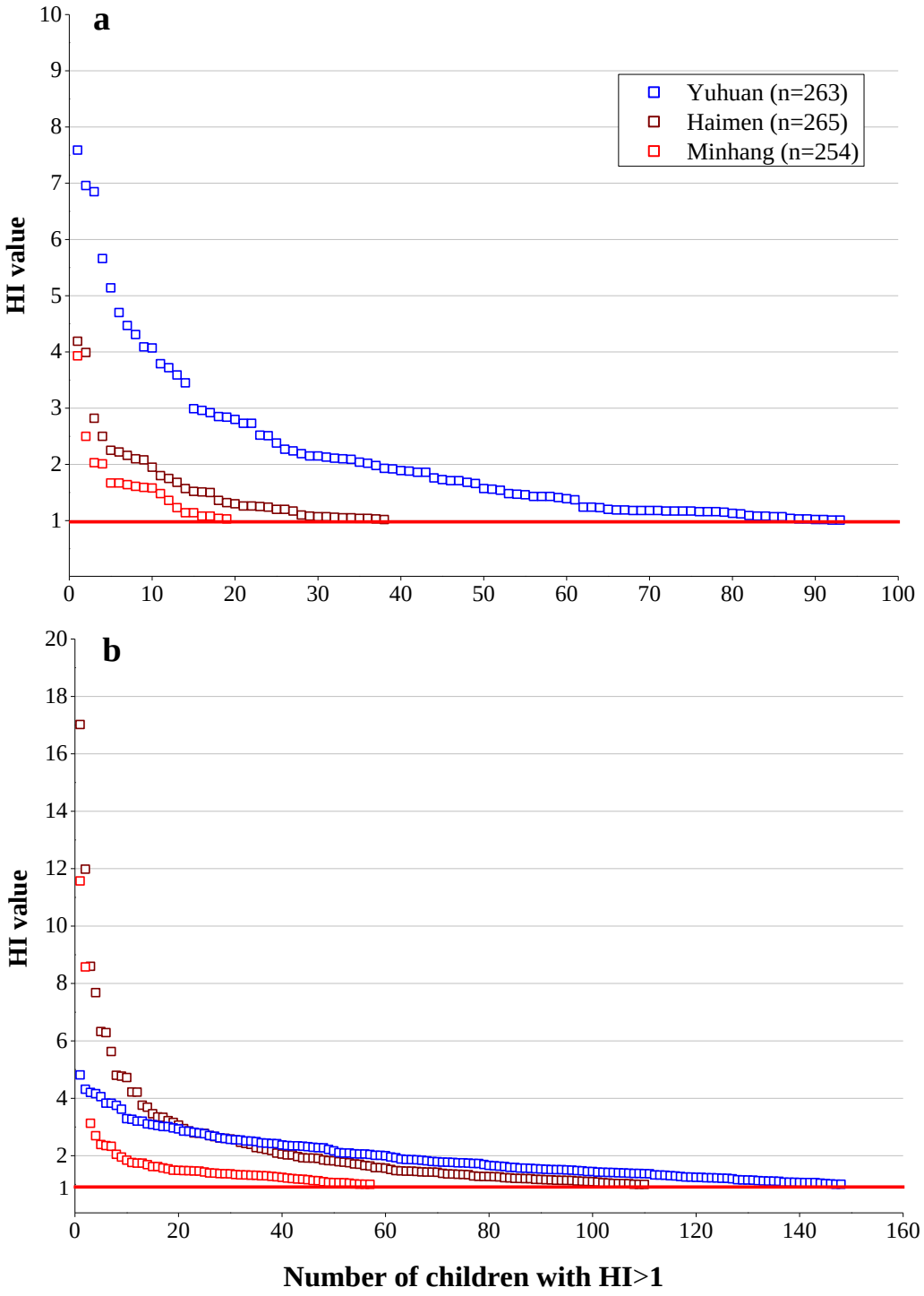

Figure S2. The distributions of HI values of the children with a HI＞1 based on EFSA's TDI in the three sampling regions of China. (a) derived from the daily intake based on volume model; (b) derived from the daily intake based on creatinine model

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