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Determination of Phthalates in Diet and Bedding for Experimental Animals Using Gas Chromatography-Mass Spectrometry

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Abstract We have developed a gas chromatography–mass spectrometry method to measure five phthalates (dibutyl phthalate, butylbenzyl phthalate, di-2-ethylhexyl phthalate, diisooctyl phthalate, and diisononyl phthalate) in diets and beddings for experimental animals. The recoveries from diets and beddings spiked with five phthalates were 98.8%–148% with coefficients of variation of 0.4%–7.8% for diets and 94.7%–146% with coefficients of variation of 1.0%–5.0% for beddings. We analyzed commercial animal diets and beddings, and found that the levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Keywords Phthalates · Animal diets · Beddings · Gas chromatography-mass spectrometry

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Phthalates are used as additives, as solvents, and as plasticizers in many consumer products. The widespread manufacture, use, and disposal of phthalates have caused ubiquitous environmental pollution and humans are regularly exposed to phthalates (Blount et al. 2000; Silva et al. 2004; Kato et al. 2005). Certain phthalates, such as dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP) and diisononyl phthalate (DINP), have been shown to disrupt development of the reproductive tract in male rodents in an antiandrogenic manner (Parks et al. 2000). Concern has been raised about phthalates in relation to effects on the reproductive tract in adult males and the development of male offspring in humans (Duty et al. 2003; Swan et al. 2005).

When animal toxicology of phthalates is studied, it is necessary to take into account the exposure from the diet and the experimental environment. Since the complete exclusion of phthalate may well be impossible, the precise concentration of phthalates in the diet, bedding and water used for feeding, the air in the experimental animal room, etc. could be important information needed to attain reliability of animal experiments. To our knowledge, no study of phthalate contamination of diets or experimental environments has been reported.

We have developed a gas chromatography-mass spectrometry (GC-MS) method to determine five commonly used phthalates (DBP, DEHP, BBzP, DINP and diisooctyl phthalate (DIOP)) in animal diets and beddings. The method was used for the analysis of commercial diets and beddings for experimental animals. Additionally, in order to investigate the other causes of rodent exposure to phthalates in feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room.

Materials and Methods

DBP, DEHP, BBzP, DIOP, DINP, DBP-3,4,5,6-d_4, DEHP-3,4,5,6-d_4, BBzP-3,4,5,6-d_4, and DOP-3,4,5,6-d_4 were purchased from Kanto Chemical (Tokyo, Japan). DNP-3,4,5,6-d_4 was purchased from Hayashi Pure Chemicals (Osaka, Japan). Phthalic acid esters and analytical grade acetonitrile, hexane, acetone and sodium chloride were purchased from Kanto Chemical. Pesticide analysis grade sodium sulfate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Bondesil-PSA (40 μm pore size) was purchased from Varian (CA, USA). Florisil® PR was purchased from Wako Pure Chemical Industries. The water used for extraction was prepared by washing distilled water with hexane.

Rodent diets were obtained from CLEA Japan Inc. (Tokyo, Japan), Oriental Yeast Co. Ltd (Tokyo, Japan), and Nihon Nosan Kogyo Co. Ltd (Tokyo, Japan). Animal beddings were obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan), Japan SLC, Inc. (Hamamatsu, Japan), CLEA Japan Inc., and Oriental Yeast Co. Ltd.

Since some of phthalates analyzed in this study are very abundant and contamination is practically unavoidable (Takatori et al. 2004), the contamination preventive measures were given as follows. No plastic apparatus was used in these experiments. The reagents and solvents were used just after opening or were not left for a long time after opening. All of the experimental apparatus, including glassware and spatulas, were washed carefully with acetone and hexane, and then heated at 200°C for 2 h to remove any phthalates. They were washed with acetone and hexane just before use. Sodium chloride, sodium sulfate and Florisil® PR were heated at 200°C for 2 h. Before starting the experiment, we took various precautionary measures, such as shortening fingernails, washing hands well with soap, covering the laboratory bench with aluminum foil, etc. A blank analysis was carried out before sample analysis in each batch. The evaporator was cleaned before use by evaporating 10 mL of acetone.

A Florisil and Bondesil-PSA column was prepared by packing Florisil (1 g), Bondesil-PSA (0.5 g) and sodium sulfate (2 g) in turn into a glass syringe (15 mm × 110 mm). The column was washed with acetone (10 mL) and hexane (10 mL) before use.

Stock solutions of native standards (DBP, DEHP, BBzP, DIOP, DINP) and isotope-labeled internal standards (DBP-3,4,5,6-d_4, DEHP-3,4,5,6-d_4, BBzP-3,4,5,6-d_4, DOP-3,4,5,6-d_4, DNP-3,4,5,6-d_4) were prepared in acetonitrile and stored at −20°C in Teflon-capped glass bottles until use. They were mixed at the desired ratio and serially diluted for calibration curves. The peak area ratio of analyte to isotope-labeled internal standard was used for quantification. Reproducible calibration curves for five phthalates were obtained with correlation coefficients greater than 0.999 (known concentration vs analyte/internal standard). They were linear over the range of 10–1,000 ng/mL for DBP, BBzP and DEHP and 50–1,000 ng/mL for DIOP and DINP.

The present method for analysis of phthalates in diets is a modification of that described by Tsumura et al. (2001). A sample of the diet (5 g) was weighed into a centrifuge tube, followed by water (5 mL) and acetonitrile (20 mL), and spiked with isotopically labeled internal standards (4 μg/mL, 25 μL). The sample was homogenized for 1 min using a homogenizer (Physcotron, Microtec Co. ltd), and then centrifuged at 3,000 rpm for 5 min. The acetonitrile layer was collected, and the residual homogenate was extracted again with 75% acetonitrile in water (20 mL). The acetonitrile layers were combined, sodium chloride (1.5 g) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was collected, hexane saturated with acetonitrile (4 mL) was added, and the mixture was shaken vigorously for 5 min. The acetonitrile layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in water (2 mL) and hexane (5 mL) and the mixture was shaken vigorously for 30 s. The solution was centrifuged at 3,000 rpm for 5 min, and the hexane layer was removed and saved. Hexane (3 mL) was added to the water layer, and extracted as described above. The hexane layers were combined and loaded onto a Florisil and Bondesil-PSA dual layer column, which was preconditioned with acetone (10 mL) and hexane (10 mL). After washing the column with hexane (3 mL), phthalates were eluted with 5% acetone in hexane (10 mL). The eluate was evaporated to dryness under reduced pressure at 35°C, and then dissolved in hexane (1 mL). An aliquot of each sample (2 μL) was injected into a GC-MS system.

The present method for analysis of phthalates in bedding is a modification of that described by Tsumura et al. (2001). A sample of bedding for experimental animals (5 g) was weighed into a centrifuge tube followed by acetone (40 mL), and spiked with isotopically labeled internal standards (4 μg/mL, 25 μL). The sample was left for 1 h and then shaken vigorously for 10 min. The solution was evaporated to dryness under reduced pressure at 35°C. The residue was treated as described for diets.

Analysis of phthalates in water was carried out as described (Glick 1998). Water (30 mL) from a tap in the experimental animal room was weighed into a centrifuge tube followed by hexane (10 mL), spiked with isotopically labeled internal standards (4 μg/mL, 25 μL), then shaken vigorously for 10 min. The hexane layer was evaporated to dryness under reduced pressure at 35°C. The residue was dissolved in hexane (1 mL) and an aliquot of each sample (2 μL) was injected into a GC-MS system.

Analysis of phthalates in air was carried out according to the method reported by the Ministry of Health, Labour and Welfare of Japan (2000). Sampling was done using an SP208-10L (GL Science, Tokyo, Japan) pump attached to an AERO cartridge SDB 400 (GL Science) with a sampling rate of 5 L/min for 24 h. After the addition of isotopically labeled internal standards (4 μg/mL, 25 μL), the collected phthalates were extracted with acetone (2 mL) by ultrasonication for 10 min. After centrifugation at 3,000 rpm for 5 min, the supernatant was collected. An aliquot of each sample (2 μL) was injected into a GC-MS system.

GC-MS analysis was performed on an Agilent 6890 N GC/5973 N MSD instrument (Agilent Technologies, CA, USA). A 30 m HP-5MS SV column (J & W Scientific, CA, USA) with 0.25 mm i.d. and 0.5 μm film thickness was used. The initial oven temperature was 80°C. After holding at the initial temperature for 2 min, the temperature was increased to 240°C at a rate of 40°C/min, and then to 300°C at a rate of 10°C/min, where it remained constant for 5 min. Helium was used as carrier gas at a flow-rate of 1.2 mL/min). The ion source temperature was 230°C and electron ionization was used as the ionization mode. The injection port was kept at 250°C. The ions used for selected ion monitoring are summarized in Table 1. DIOP was determined as the two highest peaks, and DINP as the five main peaks in the chromatogram as described by Tsumura et al. (2001).

Table 1 Retention times, quantification and confirmation ions for the measured phthalates: dibutyl phthalate, DBP; butyl benzyl phthalate, BBzP; di-2-ethylhexyl phthalate, DEHP; diisooctyl phthalate, DIOP; diisononyl

Analyte	Retention time (min)	Quantification ion	Confirmation ion
DBP	8.0	149	205, 223
DBP-d_4	8.0	153	209, 227
BBzP	10.2	149	91, 206
BBzP-d_4	10.2	153	91, 210
DEHP	11.3	149	167, 279
DEHP-d_4	11.3	153	171, 283
DIOP	11.5–12.1	149	279
DOP-d_4	12.5	153	283
DINP	12.8–13.9	149	293
DNP-d_4	14.2	153	297

Results and Discussion

The recoveries from diets and beddings spiked with 100 ng/g of DBP, BBzP and DEHP and 500 ng/g of DIOP and DINP were examined. Overall recoveries of the three repeated measurements are summarized in Table 2; these values were 98.8%–148% with coefficients of variation (CV) of 0.4%–7.8% for diets and 94.7%–146% with CV of 1.0%–5.0% for beddings. Recoveries of BBzP, DIOP and DINP were satisfactory (98.8%–113% for diets; 94.7%–119% for beddings); however, those of DBP and DEHP were relatively high (123%–148%).

The elimination of phthalate contamination is key for precise measurement. Blanks originating from sample analysis were therefore examined using phthalates-free water instead of diet and bedding. The blanks from diet sample analysis were 5.0 (±1.9) ng/g for DEHP, <3.0 ng/g for DBP and BBzP, and <20 ng/g for DIOP and DINP (n = 5), and those from bedding sample analysis were 6.6 (±2.5) ng/g for DEHP, 3.1 (±0.3) ng/g for DBP, <3.0 ng/g for BBzP, and <20 ng/g for DIOP and DINP (n = 5). The limit of quantification (LOQ) for each of five phthalates was calculated as $10S_0$, where S_0 is the value of the standard deviation obtained by analyzing quintuplicate sets of the blank analysis, or as $10S_1$, where S_1 is the value of the standard deviation obtained by analyzing quintuplicate sets of the lowest level of standard sample. The LOQ for each of five phthalates is summarized in Table 2. With our careful control of contamination, sample analysis was achieved with a low-level background, which allowed us to evaluate the amount of phthalates precisely. Typical chromatograms of a standard mixture and of diet and bedding samples are shown in Fig. 1.

Table 2 Recoveries and LOQ of phthalates

Analyte	Diet			Bedding		
	Recovery (%)	CV[a] (%)	LOQ[b] (ng/g)	Recovery (%)	CV (%)	LOQ (ng/g)
DBP	123	7.8	10	132	5.0	5
BBzP	98.8	0.4	10	102	1.0	10
DEHP	148	1.8	20	146	4.2	25
DIOP	109	0.5	50	119	4.3	50
DINP	113	1.3	50	94.7	4.3	50

Results are means of three replicate determinations

[a] *CV* coefficients of variation

[b] *LOQ* limit of quantification

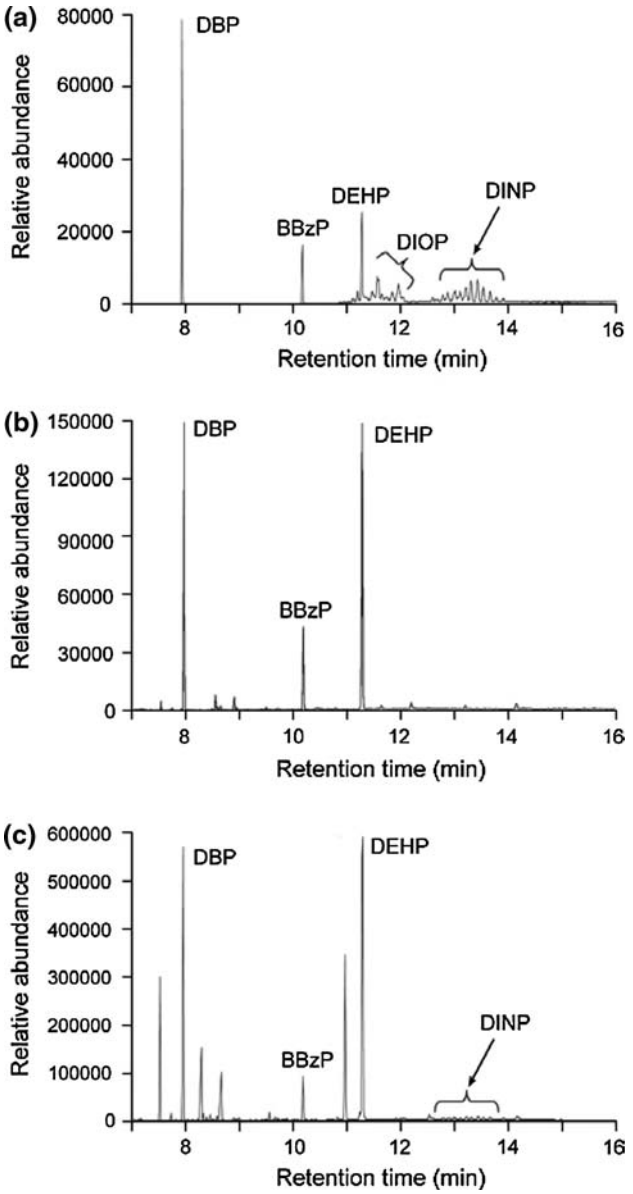

Fig. 1 Typical selected ion monitoring chromatograms of **a** a standard mixture of five phthalates, **b** diet and **c** bedding

We evaluated the suitability of the method for detecting the levels of phthalate in 12 commercial animal diets and 13 bedding materials (Table 3). All samples were analyzed just after opening the package. Two of the most frequently detected phthalates were DBP, which was found in all diets and beddings, and DEHP, which was found in all diets and in 77% of the beddings. BBzP was not detected in most samples (15%–33% detectable), and DINP was detected in only one sample of bedding. DIOP was not detected in any diet or bedding. The levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings.

Table 3 Concentrations of phthalates in commercial animal diets and beddings (ng/g)

Sample no.	DEHP	DBP	BBzP	DIOP	DINP	Sum
Diet						
D1	143	179	<LOQ	<LOQ	<LOQ	322
D2	160	46.3	<LOQ	<LOQ	<LOQ	206
D3	116	25.1	<LOQ	<LOQ	<LOQ	141
D4	511	146	157	<LOQ	<LOQ	814
D5	156	41.4	<LOQ	<LOQ	<LOQ	197
D6	146	134	<LOQ	<LOQ	<LOQ	280
D7	118	36.1	<LOQ	<LOQ	<LOQ	154
D8	205	80.4	<LOQ	<LOQ	<LOQ	285
D9	281	205	22.2	<LOQ	<LOQ	508
D10	422	403	18.4	<LOQ	<LOQ	843
D11	257	344	<LOQ	<LOQ	<LOQ	601
D12	431	944	33.4	<LOQ	<LOQ	1,410
Bedding						
B1	449	1,380	<LOQ	<LOQ	<LOQ	1,830
B2	<LOQ	757	<LOQ	<LOQ	<LOQ	781
B3	280	19.6	<LOQ	<LOQ	<LOQ	300
B4	498	765	<LOQ	<LOQ	<LOQ	1,260
B5	<LOQ	6.0	<LOQ	<LOQ	<LOQ	20.5
B6	187	128	<LOQ	<LOQ	<LOQ	315
B7	262	130	440	<LOQ	<LOQ	832
B8	420	66.4	<LOQ	<LOQ	<LOQ	486
B9	132	538	<LOQ	<LOQ	<LOQ	670
B10	5,070	1,390	900	<LOQ	198	7,560
B11	547	381	<LOQ	<LOQ	<LOQ	928
B12	443	55.5	<LOQ	<LOQ	<LOQ	499
B13	<LOQ	30.5	<LOQ	<LOQ	<LOQ	46.5

Results are means of duplicate determinations

The results of our study demonstrated that the levels of phthalates varied from sample to sample. The highest value for the concentration of five phthalates in diets (1,410 ng/g) was 10 times higher than the lowest value. If a rat weighing 150 g eats 12 g of this diet daily (Poon et al. 1997), 16,920 ng of phthalates would be ingested, which corresponds to an oral administration of 0.113 mg/kg per day. The levels of phthalates in the diets analyzed in this study are about 20–2,000 times lower than those fed in reported developmental and reproductive toxicity experiments (Poon et al. 1997; Tyl et al. 1988; Arcadi et al. 1998). However, it is necessary to take into account the progress of the contamination during storage after opening the packaging of diets.

In terms of the contamination level in beddings, one sample (no. B10), made of recycled paper, showed a remarkable level of contamination at a concentration of

Table 4 Concentrations of five phthalates in water supplied to the animals, and samples of air taken from the animal room

Sample	DEHP	DBP	BBzP	DIOP	DINP	Sum
Water (ng/mL)						
Water1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Water3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Air (ng/m^3)						
Air1	31.4	357	<LOQ	<LOQ	<LOQ	388
Air2	27.6	320	<LOQ	<LOQ	<LOQ	348
Air3	31.9	261	<LOQ	<LOQ	<LOQ	293

Results are means of duplicate determinations

five phthalates of 7,560 ng/g. Although we cannot estimate the exposure to phthalates from beddings through oral intake by licking and dermal absorption, it would be better not to use heavily contaminated bedding to avoid the potential exposure to high levels of phthalates.

In order to investigate the other causes of rodent exposure to phthalates under various feeding conditions, we analyzed water supplied to the animals, and samples of air taken from the animal room (Table 4). None of the five phthalates was detected in the water (lower limits of quantification: DBP, BBzP and DEHP, 0.5 ng/mL; DIOP and DINP, 2 ng/mL). DBP and DEHP were detected in all samples of air with concentration ranges of 261–357 ng/m^3 and 27.6–31.9 ng/m^3, respectively. Other three phthalates were not detected (lower limits of quantification: BBzP, 5 ng/m^3; DIOP and DINP, 20 ng/m^3). The contamination level in air taken from the animal room was 293–388 ng/m^3 for five phthalates. The average exposure from air amounts to 101 ng/day when we assume an average daily inhalation of 0.29 m^3 air per rat (The ICH Steering Committee 1997). This level of exposure by inhalation would be about 125 times lower than the intake from diets. These results indicate that the major source of exposure to phthalates may be the diet, although the possibility cannot be denied completely that direct incorporation of these phthalates from the lung without hydrolysis occurs.

In summary, we have developed a GC-MS method to determine five phthalates in diets and beddings used for experimental animals. We analyzed commercial animal diets and beddings, and found that both of them were polluted by phthalates, especially DBP and DEHP, suggesting that dietary exposure to phthalates routinely occurs. The total exclusion of phthalates from the experimental environment is probably impossible. Therefore, the contamination levels in the diet and bedding should be measured. Additionally, it would be wise to monitor the contamination levels of water and the air in the animal room, although the exposure from water and air was low.

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References

Arcadi FA, Costa C, Imperatore C, Marchese A, Rapisarda A, Salemi M, Trimarchi GR, Costa G (1998) Oral toxicity of bis(2-ethylhexyl) phthalate during pregnancy and suckling in the Long-Evans rat. Food Chem Toxicol 36:963–970

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ (2000) Levels of seven urinary phthalate metabolites in a human reference population. Environ Health Perspect 108:979–982

Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, Chen Z, Herrick RF, Christiani DC, Hauser R (2003) Phthalate exposure and human semen parameters. Epidemiology 14:269–277

Glick EM (1998) Liquid-liquid extraction gas chromatographic/mass spectrometric method. In: Clesceri LS, Greenberg AE, Eaton AD (eds) Standard methods for the examination of water and wastewater, 20th edn. APHA Press, North Wales, pp 6–59

Kato K, Silva MJ, Needham LL, Calafat AM (2005) Determination of total phthalates in urine by isotope-dilution liquid chromatography-tandem mass spectrometry. J Chromatogr. B 814:355–360

Ministry of Health, Labour and Welfare of Japan (2000) Committee on sick house syndrome: indoor air pollution progress report No. 1

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr (2000) The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci 58:339–349

Poon R, Lecavalier P, Mueller R, Valli VE, Procter BG, Chu I (1997) Subchronic oral toxicity of di-n-octyl phthalate and di(2-ethylhexyl)phthalate in the rat. Food Chem Toxicol 35:225–239

Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, Brock JW, Needham LL, Calafat AM (2004) Urinary levels of seven phthalate metabolites in the U.S. population from the National Health, Nutrition Examination Survey (NHANES) 1999–2000. Environ Health Perspect 112:331–338

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL, the Study for Future Families Research Team (2005) Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113:1056–1061

Takatori S, Kitagawa Y, Kitagawa M, Nakazawa H, Hori S (2004) Determination of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in human serum using liquid chromatography-tandem mass spectrometry. J Chromatogr B 804:397–401

The ICH Steering Committee. Impurities: guideline for residual solvents. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. 17 July 1997

Tsumura Y, Ishimitsu S, Saito I, Sakai H, Kobayashi Y, Tonogai Y (2001) Eleven phthalate esters and di(2-ethylhexyl) adipate in one week duplicate diet samples obtained from hospitals and their estimated daily intake. Food Addit Contam 18:449–460

Tyl RW, Price CJ, Marr MC, Kimmel CA (1988) Developmental toxicity evaluation of dietary di(2-ethylhexyl)phthalate in Fischer 344 rats and CD-1 mice. Fundam Appl Toxicol 10:395–412