Food and Chemical Toxicology 44 (2006) 1287–1298
www.elsevier.com/locate/foodchemto
doi:10.1016/j.fct.2006.02.006

Developmental toxicity of combined ethylbenzene and methylethylketone administered by inhalation to rats

A.M. Saillenfait a,*, F. Gallissot a, J.P. Sabaté a, N. Bourges-Abella b, R. Cadot c, G. Morel a, A.M. Lambert a

a Institut National de Recherche et de Sécurité , 54501 Vandoeuvre, France
b Ecole Nationale Vétérinaire de Toulouse, 31076 Toulouse Cedex 03, France
c Institut Universitaire de Médecine du Travail, 69372 Lyon Cedex 08, France

Abstract
Pregnant Sprague–Dawley rats were exposed to ethylbenzene (EB; 0, 250, or 1000 ppm) and methylethylketone (MEK; 0, 1000, or 3000 ppm), alone and in combination, by inhalation, for 6 h/day, during days 6–20 of gestation. Maternal toxicity, evidenced by decreased in body weight gain and food consumption, tended to be greater after simultaneous exposures to the high concentrations of 1000 ppm EB and 3000 ppm MEK, when compared to the treatments with individual compounds. No significant increase in embryo/fetal lethality or incidence of malformations and variations was observed in any of the treatment groups. Fetal body weight was significantly reduced after individual treatment with 1000 ppm EB or 3000 ppm MEK, and in the combined groups. There was no evidence of interaction between EB and MEK in causing developmental toxicity.

Keywords: Mixtures; Developmental toxicity; Ethylbenzene; Methylethylketone

1. Introduction
Solvents are commonly found in combinations in commercially available products (e.g., paints), and are used as mixtures in most manufacturing and processing units. A number of them have been shown to elicit developmental toxic effects in laboratory animals, but there are few data on their effects when encountered in combination (Nelson, 1994).
Ethylbenzene (EB) is an intermediate for the production of styrene and is a component of technical xylene, which is used as an industrial solvent in paints and lacquers, and in the rubber and chemical manufacturing industry. EB is also present in automotive and aviation fuels, and crude oils (WHO, 1996). There have been several studies investigating the developmental toxicity of inhaled EB in experimental animals. Hardin et al. (1981) exposed Sprague–Dawley rats and New Zealand white rabbits to 100 or 1000 ppm EB for 7 h/day, on gestation days (GD) 1–19 and 1–24, respectively.
An increased incidence of a skeletal variation (supernumerary ribs) was noted in rats at 1000 ppm EB, in the presence of maternal toxicity (i.e., increased liver, kidney and spleen weights). In a recent study, Sprague–Dawley rats were exposed to 100, 500, 1000 or 2000 ppm EB, for 6 h/ day, from GD 6 through GD 20. Maternal toxicity, evidenced by decreased food consumption and weight gain, occurred at 1000 and 2000 ppm. The fetal body weight was reduced at the two highest concentrations. Exposure to 2000 ppm also caused an increased incidence of fetuses with skeletal variations (Saillenfait et al., 2003). In contrast, post-implantation loss and fetal skeletal retardation were reported following exposure of rats to 600, 1200 and 2400 mg/m3 (i.e., 140, 280, 550 ppm, respectively), for 24 h/day, from GD 7 to GD 15 (Ungvary and Tatrai, 1985). Skeletal malformations (not specified), extra ribs, and anomalies of the uropoietic apparatus were also observed at the high concentration. In rabbits,

exposure to 1000 mg/m3 of EB (i.e., 230 ppm) for 24 h/day on GD 7–20 resulted in spontaneous abortions.

Methylethylketone (MEK) is a widely used solvent for lacquers, adhesives, and cleaning materials prior to electroplating. Little information is available in the published literature on the potential developmental toxicity of MEK. A low incidence of malformations (acaudia, imperforate anus, brachygnatia) was reported in fetuses from Sprague– Dawley rats exposed for 7 h/day to 3000 ppm MEK, from day 6 to 15 of gestation (Schwetz et al., 1974). There was also an increase in soft tissue alterations (dilated ureter and subcutaneous edema) at 3000 ppm, and in skeletal alterations (delayed ossification and extra ribs) at 1000 and 3000 ppm. No maternal toxicity was noted (i.e., serum SGPT, body and liver weight). Deacon et al. (1981) exposed Sprague– Dawley rats to 400, 1000, or 3000 ppm MEK, for 7 h/day, on GD 6–15. Increased occurrence of skeletal variations (mainly delayed ossification of skull bone) was observed at 3000 ppm, in the presence of maternal toxicity (i.e., decreased body weight gain and increased water consumption). In CD1 mice, signs of developmental toxicity were seen after exposure to 3000 ppm of MEK during major organogenesis (e.g., decrease in fetal body weight, several malformed fetuses) (Schwetz et al., 1991).

The placental transfer of EB and MEK in human pregnancy has been demonstrated (Dowty et al., 1976).

The metabolic conversion of EB proceeds mainly through oxidation of the side chain (Ensgtro¨m, 1984; Ensgtro ¨m et al., 1985). Both EB andMEK can affect the expression of particular P450 isozymes (Dietz et al., 1981; Raunio et al., 1990; Imaoka and Funae, 1991; Sequeira et al., 1992, 1994; Backes et al., 1993; Yuan et al., 1995, 1997; Bergeron et al., 1999). Metabolic interactions between EB and MEK with other industrial chemicals have been shown in rodents and humans (Angerer and Lehnert, 1979; Ensgtro¨m et al., 1984; Freundt et al., 1989; Skowron et al., 2001). Thus, combined inhalation exposure to MEK and meta-xylene or MEK and toluene resulted in inhibited oxidation of the side chain of the aromatic hydrocarbons (Liira et al., 1988, 1991; Uaki et al., 1995). MEK is known to enhance the toxicity of some other organic solvents, possibly through changes in their metabolism, e.g., the neurotoxicity of n-hexane and the hepatotoxicity of chlorinated hydrocarbons in rats.

Ethylbenzene and methylethylketone are ubiquitous environmental contaminants and have been detected in indoor air (Tang et al., 2000). They are among the most commonly used organic solvents, and are found together in several commercial and industrial applications (e.g., painting, printing, degreasing). The possibility exists that the workers and the general population may be simultaneously exposed to those two chemicals. Thus, this study was designed to examine the developmental toxic effects of EB and MEK in combination following inhalation exposure of rats during the embryonic and fetal period. Inhalation was used since it is a likely route of human exposure. To better characterize its developmental toxic effects and to aid in establishing exposure levels for the mixture study, a concentration-response study was conducted with MEK alone, which results are included in this report. The influence of simultaneous exposures to EB and MEK on common biological parameters was also examined in non-pregnant rats, using the same conditions of treatment.

2. Materials and methods

2.1. Chemicals

Ethylbenzene (EB, CAS 100-41-4, P99.5% pure) and methylethylketone (MEK, CAS 78-93-3, P99.5% pure) were obtained from Fluka Chemie AG (Buchs, Switzerland).

2.2. Animals

After two weeks of acclimatization, nulliparous female (180–200 g) Sprague–Dawley rats supplied by IFFA CREDO Breeding Laboratories (Saint-Germain-sur-l'Arbresle, France) were housed overnight with adult males (one male: two or three females) from the same strain and supplier. The day that vaginal smears were found to be sperm-positive was considered day 0 of gestation. Mated females were randomly assigned to the treatment groups using a randomization system stratified by body weight on GD 0. Mated females were housed singly in clear polycarbonate cages with stainless-steel wire lids and corn cob granules as bedding in rooms maintained at 21 ± 2 C, a relative humidity of 50 ± 5%, and a 12-h light– dark photocycle. For exposures, the females were transferred to stainlesssteel wire mesh exposure cages, and the cages were moved into the chambers. After each exposure, the animals returned to their original cages and ''home'' rooms. Food pellets (UAR Alimentation Villemoisson, France) and filtered tap water were available ad libitum except during exposures.

2.3. Experimental design

Two developmental toxicity experiments have been carried out: In the first experiment, the effects of MEK alone were investigated. Groups of 24–25 bred rats (19–23 pregnant) were exposed to 0 (control), 1000, 2000, 4000, or 6000 ppm of MEK, 6 h/day, on days 6 through 20 of gestation.

In the second experiment, the combined effects of EB and MEK were investigated. Groups of 18–19 bred rats (15–19 pregnant) were exposed to vapours of EB or MEK, separately or in combination, 6 h/day, on days 6 through 20 of gestation. There were 9 experimental groups: Control; 250 or 1000 ppm EB; 1000 or 3000 ppm MEK; or mixtures of 250 ppm EB + 1000 ppm MEK, 250 ppm EB + 3000 ppm MEK, 1000 ppm EB + 1000 ppm MEK, or 1000 ppm EB + 3000 ppm MEK. Concentrations were selected based on the results from the first experiment for MEK, and on a previous study conducted in the same laboratory for EB (Saillenfait et al., 2003). No maternal and developmental effects were expected at 250 ppm EB or 1000 ppm MEK, and mild toxicity at 1000 ppm EB or 3000 ppm MEK. Control animals were exposed concurrently to filtered room air in an adjacent chamber identical to those of the treatment groups.

2.4. Generation of test atmospheres

Exposures were conducted in 200 L glass/stainless-steel inhalation chambers with dynamic and adjustable laminar air flow (5–10 m3/h). In order to prevent any leakage of the test atmospheres, the chambers were maintained at a negative pressure of no more than 3 mm water. The chamber temperature was set at 23 ± 0.8 C and the relative humidity at 51 ± 5%. Chemical vapours were generated by passing an additional airflow rates through the fritted disk of a heated bubbler containing either of the test chemical. Under these conditions the vaporized compounds were carried out into the main air inlet pipe of the exposure chambers.

2.5. Atmosphere sampling and analysis

Concentrations of EB and MEK were monitored continuously with a gas chromatograph (Shimadzu GC14B model and Perkin Elmer autosystem XL) equipped with a flame ionization detector and an automatic gassampling valve. The column temperature was maintained at 150 C. In addition, the exposure levels were determined once during the 6-h exposure period by collecting atmosphere samples through glass tubes packed with activated charcoal. EB and MEK were then desorbed with carbon disulfide and analyzed on a Shimadzu GC-8A gas chromatograph, using toluene and n-propyl acetate as internal standards, respectively. Samples were chromatographed on a 2 m long column packed with PEG 20 M. Column and injector were maintained at 75 and 250°C, respectively. Because the concentration determined

by analyses was essentially the same as the target concentration, it will be referred to the target concentration in this paper.

2.6. Maternal and fetal evaluations

Food consumption was measured for the intervals GD 6–13 and 13– 21. Maternal body weights were recorded on GD 0, 6, 13, and 21. On GD 21, the females were killed with an intrapulmonary injection of T61 (Hoechst, Frankfurt, Germany). The uterus was then removed and weighed. The number of corpora lutea, implantation sites, resorptions, and dead and live fetuses were recorded. Uteri, which had no visible implantation sites, were stained with ammonium sulphide (10%) to detect very early resorptions (Salewski, 1964). Live fetuses were weighed, sexed, and examined for external anomalies including those of the oral cavity. Half of the live fetuses from each litter was preserved in Bouin's solution and examined for internal soft tissue changes (Wilson, 1965; Barrow and Taylor, 1969). The other half was fixed in ethanol (70%), eviscerated, and then processed for skeletal staining with alizarin red S for subsequent skeletal examination (Staples and Schnell, 1964).

2.7. Short-term toxicity study on EB and MEK mixture

Because of the paucity of data on EB and MEK combinations, additional toxic and metabolic endpoints were examined to help characterize possible interaction between the two solvents. Complementary data were also needed to better understand the factors that may determine the effects of the mixture.

In a separate experiment, groups of 6 non-pregnant female rats were exposed to EB and MEK, alone and in combination, for 15 days, under the same experimental conditions as the pregnant rats. At the end of the first and last exposures, the rats were placed in individual metabolic cages in which they had free access to food and water, and urines were collected at +4 C over a 16-h period. After the last urine collection, the animals were euthanized by bleeding from the abdominal aorta under anaesthesia. The liver and the kidney were removed, weighed and prepared for histologic evaluation: They were fixed in 10% neutral buffered formalin prior to paraffin embedding. Sections were cut at 3 lm and stained with haematoxylin and eosin (Ruehl-Fehlert et al., 2003).

Biochemical parameters were measured in the samples of serum and urine using an automated analyzer (Cobas Mira Plus, Roche Diagnostics) and reagent kits (Roche Diagnostics, ABX Diagnostics). Serum endpoints included: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea, creatinine. Urine evaluations included: volume, creatinine, proteins, and enzyme activities of c-glutamyl transferase (GGT), alkaline phosphatase (ALP), and lactate deshydrogenase (LDH).

In addition, aliquots of urine were frozen at -20°C until determination of mandelic acid according to a modification of the HPLC methods of Kivisto et al. (1993) and Ogata and Sugihara (1978).

2.8. Statistical analysis

Whenever possible, the data were presented as mean ± SD. The litter was used as the basis for analysis of fetal variables.

2.8.1. Developmental toxicity study on MEK alone

The number of implantation sites and live fetuses and various body weights were analyzed by one-way analysis of variance, followed by Dunnett's test if differences were found. The frequency of post-implantation loss, dead fetuses, resorptions and alterations among litters was evaluated by using the Kruskal–Wallis test followed by the Mann–Whitney test where appropriate. Rates of pregnancy, and proportions of fetuses and litters with any

malformations, or external, visceral, skeletal, or any variations in the treated groups were compared with those of the control group by the Fisher's test. Where applicable, least-squares analysis was carried out. The reported level of statistical significance was $p < 0.05$.

2.8.2. Studies on EB and MEK mixture

A large number of data sets did not show homogeneity of variance (Bartlett's test). Therefore, all data were analyzed using a non-parametric analysis of variance (Kruskal–Wallis test), followed by the Mann–Whitney test for multiple comparisons among the treatment groups, when significance was indicated ($p < 0.05$). Interactions between EB and MEK were analyzed by two-way analysis of variance on ranks. Additivity was assumed when this analysis showed no significant interaction between the two solvents ($p > 0.1$).

3. Results

3.1. Developmental toxicity study on MEK alone

No test dam died (Table 1). Maternal weight gain was decreased during the first half of exposure at 4000 ppm, and during the whole exposure period at 6000 ppm (Table 1). The corrected weight gain was significantly depressed at 4000 and 6000 ppm. Food consumption was significantly reduced throughout the exposure period at 4000 and 6000 ppm (Table 2). The number of implantations and of live fetuses, and the incidence of non-live implants and resorptions were comparable across groups (Table 3). There was a concentration-related decrease in fetal body weight (all, males, and females), which achieved statistical significance at 2000 ppm and above. The 4% decrease at 2000 ppm reached 19–20% at 6000 ppm. Malformations occurred in single instances at the two highest concentrations of MEK (Table 4). At 4000 ppm, they included one fetus with unilateral anophthalmia, one fetus with diaphragmatic hernia, and one fetus with anal atresia, thread-like tail, and various malformations of the vertebral column (fused thoracic archs and centra, sacral and caudal vertebrae absent). Anal atresia associated with absence of tail was also present in one fetus from the 6000 ppm MEK group. No variation was observed externally. Visceral variations (mainly dilated ureter) were distributed across all groups, with no indication of any treatmentrelated effect. The incidences of two skeletal variations (i.e., delayed ossification of sternebrae and rudimentary cervical ribs) were elevated at 4000 and 6000 ppm (Table 5) However, there were no significant changes in the incidence of total skeletal variations or all variations.

Table 1
Body weight gain of dams inhaling methylethylketone on days 6–20 of gestation

Treatment (ppm/6 h/day)		No. dams	Body weight gain (g) on GD				
			0–6	6–13	13–21	6–21	Corrected weight gain[b]
0		21	33 ± 7[a]	31 ± 8	105 ± 21	137 ± 24	31 ± 11
MEK	1000	23	33 ± 9	32 ± 6	106 ± 20	137 ± 23	30 ± 11
	2000	19	31 ± 9	32 ± 7	106 ± 15	138 ± 18	33 ± 12
	4000	21	33 ± 7	15 ± 9**	94 ± 13	109 ± 15**	15 ± 12**
	6000	21	33 ± 8	10 ± 8**	87 ± 14**	97 ± 15**	7 ± 12**

Significantly different from control group (air), $^{**}p < 0.01$.
[a] Values are expressed as mean ± SD.
[b] Body weight gain on GD 6-21 minus gravid uterine weight.

Table 2
Food consumption of dams inhaling methylethylketone on days 6–20 of gestation

Treatment (ppm/6 h/day)		No. dams	Food consumption (g/dam/day) on GD			
			0–6	6–13	13–21	6–21
0		21	22 ± 2[a]	23 ± 2	27 ± 2	25 ± 2
MEK	1000	23	22 ± 3	23 ± 2	27 ± 3	25 ± 2
	2000	19	22 ± 2	24 ± 2	28 ± 2	26 ± 2
	4000	21	22 ± 2	19 ± 2**	25 ± 2*	22 ± 2**
	6000	21	22 ± 2	17 ± 2**	24 ± 2**	21 ± 2**

Significantly different from control group (air), $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.

Table 3
Reproductive parameters in rats inhaling methylethylketone on days 6–20 of gestation

Treatment (ppm/6 h/day)		No. pregnant/treated	Implantations/litter	% non-live implants/litter	% resorptions/litter	Live fetus/litter	Fetal body weight (g)		
							All	Males	Females
0		21/25	14.2 ± 3.4[a]	4.3 ± 5.3	4.3 ± 5.3	13.6 ± 3.4	5.87 ± 0.28	6.03 ± 0.32	5.68 ± 0.25
MEK	1000	23/25	14.7 ± 2.8	5.1 ± 4.9	5.1 ± 4.9	14.1 ± 2.8	5.68 ± 0.31	5.86 ± 0.32	5.52 ± 0.32
	2000	19/25	15.3 ± 2.6	7.8 ± 9.1	7.8 ± 9.1	14.2 ± 2.9	5.61 ± 0.31*	5.79 ± 0.29*	5.44 ± 0.33*
	4000	21/24	14.7 ± 2.5	4.5 ± 4.7	4.2 ± 4.4	14.1 ± 2.4	4.96 ± 0.30**	5.11 ± 0.31**	4.82 ± 0.29**
	6000	21/24	15.4 ± 2.1	8.4 ± 7.2	8.4 ± 7.2	14.1 ± 2.3	4.71 ± 0.34**	4.87 ± 0.31**	4.53 ± 0.34**

Significantly different from control group (air), $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.

3.2. Developmental toxicity study on EB and MEK mixture

3.2.1. Maternal effects

Exposure to EB or MEK alone did not cause any significant changes in maternal body weight gain compared with the control, although a slight decrease was observed during GD 6–13 in rats inhaling 1000 ppm EB (Table 6). In contrast, a significant reduction occurred on GD 6–13 after co-exposure to 250 ppm EB and 3000 ppm MEK, and during the entire treatment period after combined exposure to 1000 ppm EB with 1000 or 3000 ppm MEK. Corrected weight gain was significantly less than control in groups receiving 1000 ppm EB or 3000 ppm MEK, alone or in combination with either levels of the other solvent.

Food consumption was significantly lower than control on GD 13–21 in the low concentration mixture group (250 ppm EB and 1000 ppm MEK), and throughout exposure in the three other mixture groups (Table 7). When EB and MEK were given alone, food consumption was only significantly different from control at 1000 ppm EB, on GD 6–13. Statistical analysis indicated a significant interaction for maternal weight gain on GD 6–13 ($p < 0.01$) and GD 6–21 ($p < 0.06$), and for maternal food consumption on GD 6–13 ($p < 0.1$).

3.2.2. Reproductive and fetal effects

EB and MEK, alone or in combination, have no effect on the average number of implantations and live fetuses, and in the incidence of non-live implants and resorptions (Table 8). The body weight of the fetuses (all, males, females) was significantly lower than control after exposure to the high concentrations of EB or MEK alone (1000 and 3000 ppm, respectively), or to either mixtures. No statisti- cally significant interaction was found, and when the decreases associated with exposure to mixtures of EB and MEK were compared with the decreases observed after exposure to individual solvents, additivity was indicated. Thus, they amounted 8% at 1000 ppm EB, 6–7% at 3000 ppm MEK, and 18–19% following co-exposure to 1000 ppm EB with 3000 ppm MEK. Few malformations were seen, which were limited to the 1000 ppm EB group (visceral) and to the four combination groups (external, visceral and/or skeletal) (Table 9). Each finding occurred in no more than one or two fetuses per group. These malformations could not be conclusively attributed to treatment, since the incidences of specific or total malformations were not significantly different from control, and did not show

a concentration-related pattern. Common external, visceral, and skeletal variations (primarily 14th ribs and/or incomplete ossification of thoracic vertebral centra) were seen, with no significant differences in their individual or total incidences between the control and treated groups (Table 10).

Table 4
Observations in fetuses of rats inhaling methylethylketone on days 6–20 of gestation (summary)

Treatment (ppm/6 h/day)	0	MEK 1000	MEK 2000	MEK 4000	MEK 6000
No. fetus (litter) examined					
External	285 (21)	324 (23)	269 (19)	295 (21)	297 (21)
Skeletal	143 (21)	162 (23)	134 (19)	148 (21)	149 (21)
Visceral	142 (21)	162 (23)	135 (19)	147 (21)	148 (21)
Total with any malformations					
No. fetuses (litters)	0	0	0	3 (3)	1 (1)
Mean %/litter	0	0	0	1.1 ± 2.8[a]	0.4 ± 1.7
Total with external variations					
No. fetuses (litters)	0	0	0	0	0
Mean %/litter	0	0	0	0	0
Total with visceral variations					
No. fetuses (litters)	13 (6)	4^{*} (4)	6 (5)	6 (3)	12 (7)
Mean %/litter	9.9 ± 19.8	2.1 ± 4.8	4.2 ± 7.7	4.9 ± 13.2	8.6 ± 15.0
Total with skeletal variations					
No. fetuses (litters)	32 (14)	34 (16)	25 (12)	48 (17)	48 (17)
Mean %/litter	20.8 ± 19.0	21.4 ± 22.4	19.6 ± 21.0	32.6 ± 25.5	32.5 ± 25.5
Total with any variations					
No. fetuses (litters)	45 (14)	38 (17)	31 (16)	54 (17)	60 (18)
Mean %/litter	15.0 ± 13.7	12.0 ± 12.0	11.8 ± 9.0	18.6 ± 14.9	20.2 ± 15.7

Significantly different from control group (air), $^{*}p < 0.05$.
[a] Values are expressed as mean ± SD.

Table 5
Incidences of specific skeletal variations in fetuses of rats inhaling methylethylketone on days 6–20 of gestation[a]

Treatment (ppm/6 h/day)	0	MEK 1000	MEK 2000	MEK 4000	MEK 6000
Hyoid, incomplete ossification	1 (1)	0	0	0	0
Sternebrae, incomplete ossification	4 (3)	4 (4)	5 (3)	17 $(12)^{**}$	23 $(13)^{**}$
Sternebrae fused (first and second)	0	1 (1)	0	0	0
Ribs, cervical rudimentary	1 (1)	2 (2)	5 (3)	12 (8)	11 (6)
Ribs, 14th supernumerary	17 (9)	4 (2)	3 (3)	20 (10)	12 (7)
Ribs, incomplete ossification (13 or 14th)	0	0	1 (1)	1 (1)	0
Thoracic vertebral centra, incomplete ossification	10 (4)	26 (12)	12 (6)	8 (6)	11 (10)

Significantly different from control group (air), $^{**}p < 0.01$.
[a] Number of fetuses (number of litters) affected.

Table 6
Body weight gain of dams inhaling ethylbenzene and methylethylketone, alone or in combinations, on days 6–20 of gestation
Treatment

Treatment (ppm/6 h/day)		No. dams	Body weight gain (g) on GD				
			0–6	6–13	13–21	6–21	Corrected weight gain[b]
0		16	30 ± 7[a]	30 ± 6	94 ± 33	123 ± 36	34 ± 8
EB	250	19	31 ± 7	27 ± 9	109 ± 19	136 ± 21	27 ± 16
	1000	18	30 ± 7	23 ± 12	97 ± 22	120 ± 29	$18 \pm 18^{**}$
MEK	1000	18	33 ± 9	31 ± 5	106 ± 26	137 ± 29	32 ± 13
	3000	18	35 ± 7	29 ± 7	102 ± 12	130 ± 14	$25 \pm 15^{*}$
EB/MEK	250/1000	17	33 ± 10	30 ± 8	101 ± 18	131 ± 20	27 ± 12
	250/3000	18	34 ± 8	$24 \pm 6^{*,\ddagger}$	$96 \pm 16^{\dagger}$	$120 \pm 19^{\dagger}$	$22 \pm 9^{**}$
	1000/1000	17	30 ± 9	$15 \pm 9^{**,\dagger,\ddagger\ddagger}$	$79 \pm 28^{*,\dagger,\ddagger\ddagger}$	$95 \pm 32^{**,\dagger,\ddagger\ddagger}$	$13 \pm 22^{**,\ddagger\ddagger}$
	1000/3000	15	32 ± 5	$8 \pm 9^{**,\dagger\dagger,\ddagger\ddagger}$	$80 \pm 25^{*,\dagger,\ddagger\ddagger}$	$88 \pm 26^{**,\dagger\dagger,\ddagger\ddagger}$	$10 \pm 12^{**,\dagger,\ddagger\ddagger}$

Significantly different from control group (air), $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively. Significantly different from EB alone at the same concentration, $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, respectively. Significantly different from MEK alone at the same concentration, $^{\ddagger}p < 0.05$ and $^{\ddagger\ddagger}p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.
[b] Body weight gain on GD 6-21 minus gravid uterine weight.

Table 7
Food consumption of dams inhaling ethylbenzene and methylethylketone, alone or in combinations, on days 6–20 of gestation

Treatment (ppm/6 h/day)		No. dams	Food consumption (g/dam/day) on GD			
			0–6	6–13	13–21	6–21
0		16	24 ± 2^{a}	24 ± 2	27 ± 3	26 ± 2
EB	250	19	24 ± 2	23 ± 3	28 ± 4	25 ± 3
	1000	18	23 ± 2	$20 \pm 2^{**}$	26 ± 3	$23 \pm 3^{**}$
MEK	1000	18	24 ± 2	24 ± 2	28 ± 2	26 ± 2
	3000	18	24 ± 3	23 ± 2	28 ± 2	26 ± 2
EB/MEK	250/1000	17	23 ± 3	23 ± 3	$26 \pm 3^{*,\ddagger}$	$25 \pm 3^{\ddagger}$
	250/3000	18	23 ± 3	$21 \pm 2^{**,\dagger,\ddagger\ddagger}$	$26 \pm 2^{*,\dagger\ddagger}$	$23 \pm 3^{**,\dagger,\ddagger\ddagger}$
	1000/1000	17	23 ± 3	$18 \pm 3^{**,\dagger,\ddagger\ddagger}$	$23 \pm 2^{**,\dagger,\ddagger\ddagger}$	$21 \pm 2^{**,\dagger,\ddagger\ddagger}$
	1000/3000	15	23 ± 2	$17 \pm 2^{**,\dagger\dagger,\ddagger\ddagger}$	$24 \pm 3^{**,\ddagger\ddagger}$	$20 \pm 2^{**,\dagger\dagger,\ddagger\ddagger}$

Significantly different from control group (air), $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively. Significantly different from EB alone at the same concentration, $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, respectively. Significantly different from MEK alone at the same concentration, $^{\ddagger}p < 0.05$ and $^{\ddagger\ddagger}p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.

Table 8
Reproductive parameters in rats inhaling ethylbenzene and methylethylketone, alone or in combinations, on days 6–20 of gestation

Treatment (ppm/6 h/day)		No. pregnant/ treated	Implan- tations/ litter	% non-live implants/ litter	% resorptions/ litter	Live fetus/ litter	Fetal body weight (g)		
							All	Males	Females
0		16/19	12.1 ± 4.8^{a}	12.8 ± 26.5	12.8 ± 26.5	11.4 ± 5.0	5.90 ± 0.34	6.04 ± 0.35	5.70 ± 0.31
EB	250	19/19	15.1 ± 1.9	5.5 ± 4.7	5.1 ± 4.9	14.3 ± 2.1	5.70 ± 0.45	5.85 ± 0.51	5.55 ± 0.45
	1000	18/19	14.4 ± 3.5	2.4 ± 4.6	2.4 ± 4.6	14.1 ± 3.6	$5.42 \pm 0.34^{**}$	$5.57 \pm 0.33^{**}$	$5.26 \pm 0.35^{**}$
MEK	1000	18/18	13.8 ± 4.1	4.7 ± 12.0	4.7 ± 12.0	13.5 ± 4.3	5.82 ± 0.31	5.91 ± 0.23	5.66 ± 0.36
	3000	18/19	14.8 ± 2.7	4.2 ± 4.5	4.2 ± 4.5	14.2 ± 2.7	$5.48 \pm 0.30^{**}$	$5.60 \pm 0.30^{**}$	$5.34 \pm 0.32^{**}$
EB/MEK	250/1000	17/19	14.2 ± 2.7	$1.2 \pm 2.6^{\dagger\dagger}$	$1.2 \pm 2.6^{\dagger}$	14.0 ± 2.7	$5.55 \pm 0.26^{**,\ddagger}$	$5.72 \pm 0.25^{**}$	$5.38 \pm 0.30^{*,\ddagger}$
	250/3000	18/19	14.4 ± 1.9	7.3 ± 12.2	7.3 ± 12.2	13.5 ± 2.7	$5.39 \pm 0.24^{**,\dagger\dagger}$	$5.56 \pm 0.29^{**,\dagger}$	$5.25 \pm 0.23^{**,\dagger\dagger}$
	1000/1000	17/19	12.4 ± 4.7	5.4 ± 5.2	5.4 ± 5.2	11.6 ± 4.4	$5.32 \pm 0.36^{**,\ddagger\ddagger}$	$5.44 \pm 0.36^{**,\ddagger\ddagger}$	$5.18 \pm 0.38^{**,\ddagger\ddagger}$
	1000/3000	15/18	13.9 ± 3.5	10.9 ± 25.2	10.9 ± 25.2	12.4 ± 4.7	$4.78 \pm 0.33^{**,\dagger\dagger,\ddagger\ddagger}$	$4.94 \pm 0.29^{**,\dagger\dagger,\ddagger\ddagger}$	$4.67 \pm 0.37^{**,\dagger\dagger,\ddagger\ddagger}$

Significantly different from control group (air), $^{*}p < 0.05$ and $^{**}p < 0.01$, respectively. Significantly different from EB alone at the same concentration, $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$, respectively. Significantly different from MEK alone at the same concentration, $^{\ddagger}p < 0.05$ and $^{\ddagger\ddagger}p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.

3.3. Short-term toxicity study on EB and MEK mixture

3.3.1. Serum and urine biochemistry

In non-pregnant rats, repeated co-exposures to the high concentration mixture (1000 ppm EB and 3000 ppm MEK) resulted in a 2- to 2.6-fold increase in the urinary excretion of ALP ($p < 0.05$), LDH, creatinine ($p < 0.01$), and proteins, relative to control. The urinary volume was also significantly elevated (22.2 ml versus 6.1 ml in control, $p < 0.01$), as were serum ALAT, ASAT (Table 11). 3.3.2. Liver and kidney weights and histopathology Compared with control, both absolute liver weight and liver to body weight ratios were significantly elevated in animals treated with 250 ppm EB, 1000 ppm EB, or 3000 ppm MEK, alone, or with either mixtures (Fig. 1). The magnitude of increase was slightly higher in rats coexposed to 1000 ppm EB and 3000 ppm MEK, compared to the changes associated with individual EB and MEK (30%, 13–16%, and 65–70%, for 1000 ppm EB, 3000 ppm MEK, and combined 1000 ppm EB and 3000 ppm MEK, respectively). However, no statistically significant interaction was found.

Table 9
Types and incidences of malformations in fetuses of rats inhaling ethylbenzene and methylethylketone, alone or in combinations, on days 6–20 of gestation[a]

Treatment (ppm/6 h/day)		0	EB 250	EB 1000	MEK 1000	MEK 3000	EB/MEK 250/1000	EB/MEK 250/3000	EB/MEK 1000/1000	EB/MEK 1000/3000
No. fetus (litter) examined	External	182 (15)	272 (19)	253 (18)	243 (18)	255 (18)	238 (17)	243 (18)	197 (17)	186 (14)
	Skeletal	91 (15)	136 (19)	127 (18)	122 (18)	127 (18)	119 (17)	122 (18)	100 (17)	93 (14)
	Visceral	91 (15)	136 (19)	126 (18)	121 (17)	128 (18)	119 (17)	121 (18)	97 (17)	93 (14)
Micrognatia		0	0	0	0	0	0	1 (1)[b]	0	0
General oedema		0	0	0	0	0	1 (1)[c]	0	0	0
Anal atresia, and tail thread like and/or short		0	0	0	0	0	2 (2)[cd]	0	0	0
Cerebrum misshapen		0	0	0	0	0	0	1 (1)	0	0
Transposed aorta, interventricular septum defect		0	0	0	0	0	1 (1)	0	0	0
Diaphragmatic hernia		0	0	2 (2)	0	0	1 (1)	1 (1)	0	2 (2)
Sternebrae, multiple fusions and misshapen		0	0	0	0	0	0	0	1 (1)	0
Cervical arches fused		0	0	0	0	0	0	1 (1)	0	0
Thoracic vertebrae: arch absent, hemicentra		0	0	0	0	0	0	1 (1)[b]	0	0
Multiple ribs and vertebrae malformations		0	0	0	0	0	2 (2)[cd]	0	0	0
Total with any malformations	No.	0	0	2 (2)	0	0	4 (2)	4 (4)	1 (1)	2 (2)
	Mean %/litter	0	0	0.9 ± 2.5[e]	0	0	1.7 ± 4.8	1.6 ± 3.2	0.3 ± 1.4	1.0 ± 2.4

[a] Number of fetuses (number of litters) affected.
[b,c,d] Findings with the same letter subscript are from the same fetus.
[cd] Thoracic, lumbar and/or sacral vertebrae absent, and ribs absent. Thoracic centra fused.
[e] Mean ± SD.

There was no significant change in the absolute and relative kidney weight whatever treatment group. Histological evaluation of liver and kidney revealed no pathological effects attributable to individual or combined solvents exposures. Periportal hepatocyte hypertrophy with clear cytoplasm, compared with centrilobular hepatocytes, was commonly noted. This observation was minimally present in the control group and appeared moderate to marked in the high concentrations groups. Findings of this nature are in favour of an adaptative response without pathological signification (Greaves, 2000).

3.3.3. Urine mandelic acid levels

The excretion of mandelic acid was 2- to 5-fold higher after 15 exposures than after one exposure to 1000 ppm EB, alone or in combination. After repeated treatments, the urinary level of mandelic acid was significantly greater after co-exposure to 1000 ppm EB and 3000 ppm MEK, compared with 1000 ppm EB alone (Fig. 2). A significant EB by MEK interaction was seen ($p < 0.01$). No other significant difference was noted in mixtures when compared to animals treated with EB alone, although mandelic acid tended to decrease with increasing concentration of MEK, after one exposure to EB at either concentration or after repeated exposures to 250 ppm EB ($p < 0.1$).

4. Discussion

The main purpose of this investigation was to evaluate the potential for interaction between the two solvents, EB and MEK, on the prenatal development in the rat. At the levels used in our study, EB and MEK alone caused no or limited maternal effects, while evidence of maternal toxicity was observed after concurrent exposures to increasing concentrations EB and MEK, as reflected by decreases in maternal weight gain, corrected body weight, and/or food consumption. Interactions between EB and MEK were demonstrated for several maternal endpoints, including weight gain and food consumption on GD 6–13. Their decreases were greater for combined EB and MEK than the summation of individual responses, thereby the mixture produced effects seemingly greater than additive (Nelson,

1994; Haghdoost et al., 1997). Nevertheless, a minimal level (1000 ppm EB and/or 3000 ppm MEK) was considered necessary for enhancement since no change was seen in animals exposed to the lowest concentration of mixture (i.e., 250 ppm EB with 1000 ppm MEK). The developmental toxic effects of the mixture were consistent with the effects of the individual solvents. Thus, exposure to EB and MEK either alone or in combination resulted in fetal toxicity, as evidenced by decreased fetal body weight, and there was no increase in embryolethality and in the incidence of fetal malformations. The magnitude of fetal weight decreases induced by co-administration of EB with MEK was not greater than the additive responses of the individual chemicals. Thus, combination effects vary with different endpoints within the same experimental design: The two solvents working synergically on several maternal parameters showed no significant interaction on the developmental endpoints assessed. It is noticeable that the interactions were mostly evident at the highest concentrations of mixture. In a risk assessment situation, the default assumption of response additivity may not underestimate maternal and developmental toxic effects caused by concurrent inhalation exposures to low levels of EB and MEK (ATSDR, 2004). However, responses may vary with the treatment conditions (e.g., period, route).

Table 10
Types and incidences of variations malformations in fetuses of rats inhaling ethylbenzene and methylethylketone, alone or in combinations on days 6–20
of gestation[a]

Treatment (ppm/6 h/day)		0	EB 250	EB 1000	MEK 1000	MEK 3000	EB/MEK 250/1000	EB/MEK 250/3000	EB/MEK 1000/1000	EB/MEK 1000/3000
No. fetus (litter) examined	External	182 (15)	272 (19)	253 (18)	243 (18)	255 (18)	238 (17)	243 (18)	197 (17)	186 (14)
	Skeletal	91 (15)	136 (19)	127 (18)	122 (18)	127 (18)	119 (17)	122 (18)	100 (17)	93 (14)
	Visceral	91 (15)	136 (19)	126 (18)	121 (17)	128 (18)	119 (17)	121 (18)	97 (17)	93 (14)
Club foot (unilateral)		0	0	0	0	0	1 (1)	0	0	0
Dilated renal pelvis		0	0	1 (1)	2 (2)	0	0	1 (1)	0	0
Distended ureter		8 (5)	5 (3)	10 (8)	6 (6)	5 (5)	5 (3)	8 (6)	10 (6)	7 (4)
Testis slightly displaced		0	0	0	0	0	1 (1)	0	0	0
Sternebrae, incomplete ossification		1 (1)	7 (4)	1 (1)	5 (5)	7 (4)	1 (1)	3 (2)	2 (2)	2 (1)
Sternebrae fused (second and third)		0	0	0	0	0	1 (1)	0	0	0
Ribs, cervical rudimentary		1 (1)	0	0	3 (3)	9 (6)	1 (1)	0	3 (2)	3 (3)
Ribs, 14th supernumerary		16 (8)	33 (15)	28 (10)	10 (7)	28 (12)	15 (5)	18 (9)	20 (8)	17 (6)
Thoracic vertebral centra, incomplete ossification		6 (4)	9 (7)	4 (3)	6 (3)	9 (6)	14 (7)	7 (4)	6 (4)	14 (6)
Total with external variations	No.	0	0	0	0	0	1 (1)	0	0	0
	Mean %/litter	0	0	0	0	0	0.3 ± 1.4	0	0	0
Total with visceral variations	No.	8 (5)	5 (3)	10 (8)	6 (6)	5 (5)	6 (4)	8 (6)	10 (6)	7 (4)
	Mean %/litter	8.1 ± 13.8[b]	4.3 ± 10.8	9.1 ± 13.4	10.1 ± 23.8	4.0 ± 6.7	4.6 ± 10.9	6.7 ± 12.6	13.5 ± 26.1	6.6 ± 12.3
Total with skeletal variations	No.	24 (11)	43 (16)	31 (12)	21 (11)	46 (18)	28 (9)	25 (12)	29 (11)	30 (11)
	Mean %/litter	23.0 ± 18.9	30.9 ± 20.8	23.0 ± 24.9	18.3 ± 16.6	37.8 ± 19.5	24.9 ± 33.0	19.4 ± 21.3	27.8 ± 28.5	31.7 ± 29.8
Total with any variations	No.	32 (12)	48 (16)	41 (14)	27 (13)	51 (18)	34 (11)	33 (13)	39 (14)	37 (13)
	Mean %/litter	15.6 ± 13.2	17.6 ± 11.8	16.8 ± 14.1	16.3 ± 22.8	20.7 ± 9.0	14.7 ± 16.5	12.7 ± 13.5	20.8 ± 16.8	19.8 ± 14.8

[a] Number of fetuses (number of litters) affected.
[b] Mean ± SD.

Table 11
Serum chemistry of non-pregnant rats inhaling ethylbenzene and methylethylketone, alone or in combinations

Treatment (ppm/6 h/day)		ALAT (IU/l)	ASAT (IU/l)	Urea (g/l)	Creatinine (mg/l)
0		21 ± 3[a]	67 ± 6	0.31 ± 0.06	4.95 ± 0.55
EB	250	20 ± 3	65 ± 4	0.34 ± 0.04	4.82 ± 0.56
	1000	22 ± 2	64 ± 10	0.34 ± 0.07	4.63 ± 0.34
MEK	1000	20 ± 2	69 ± 12	0.33 ± 0.03	5.25 ± 0.74
	3000	24 ± 5	74 ± 10	0.34 ± 0.07	5.10 ± 0.37
EB/MEK	250/1000	21 ± 6	64 ± 7	0.34 ± 0.06	5.27 ± 0.89
	250/3000	29 ± 6*,†	78 ± 12†	0.32 ± 0.05	5.20 ± 0.51
	1000/1000	27 ± 7‡‡	75 ± 10	0.34 ± 0.03	4.90 ± 0.41
	1000/3000	44 ± 6**,††,‡‡	91 ± 13*,††,‡	0.34 ± 0.03	5.05 ± 0.24

Significantly different from control group (air), *$p < 0.05$ and **$p < 0.01$, respectively. Significantly different from EB alone at the same concentration, †$p < 0.05$ and ††$p < 0.01$, respectively. Significantly different from MEK alone at the same concentration, ‡$p < 0.05$ and ‡‡$p < 0.01$, respectively.
[a] Values are expressed as mean ± SD.

Fig. 1. Liver-body weight ratios. Significantly different from control group (air), *p < 0.05 and **p < 0.01, respectively. Significantly different from EB
alone at the same concentration, _p < 0.01. Significantly different from MEK alone at the same concentration, _p < 0.01.

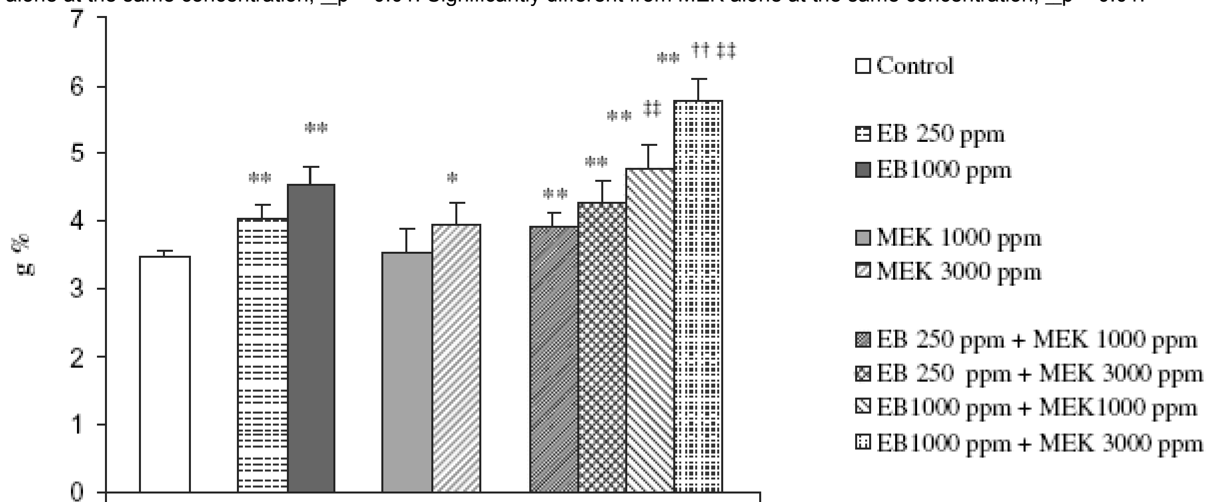
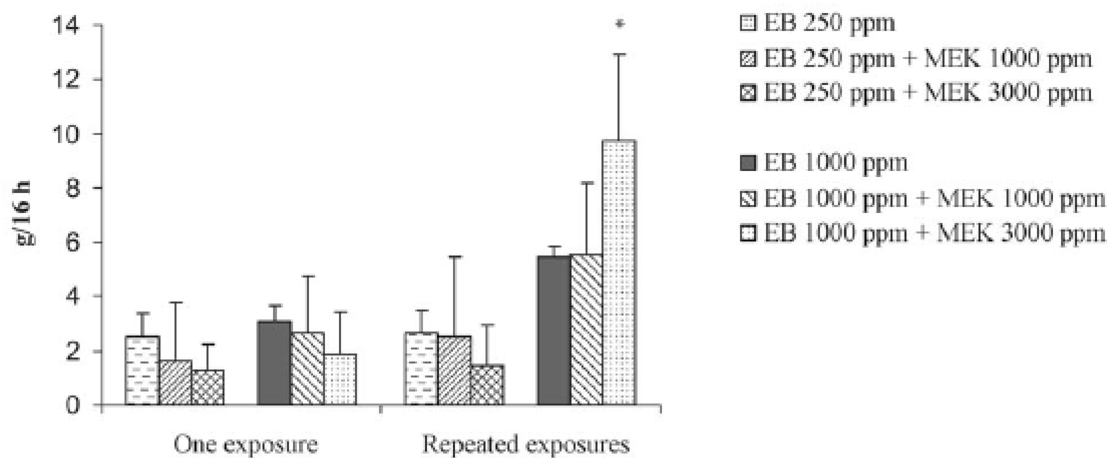


Fig. 2. Urinary excretion of mandelic acid. Significantly different from ethylbenzene alone at the same concentration, *p < 0.05.



Our developmental toxicity findings agree with previous results in rats given EB alone under similar exposure conditions (e.g., length of daily exposure) (Hardin et al., 1981; Saillenfait et al., 2003). Schwetz et al. (1974) have reported a low incidence of anal atresia associated with tail defects after exposure of rats to 3000 ppm MEK, 7 h/day, on GD 6–15. Sporadic cases of

these malformations were observed in our dose-range finding study at 4000 and 6000 ppm MEK in the presence of maternal toxicity, and after co-exposure to 250 ppm EB with 1000 ppm MEK. However, their occurrence remained rare, with no clear dose-response relationship.

Similarly the developmental toxicity study, additional evaluation of the effects of the mixture on some toxic and metabolic endpoints in adult female animals revealed minimal interaction between EB and MEK at the low concentrations levels. Only prolonged exposure to the mixture at high concentrations led to more consistent changes. Thus, individual treatment to EB or MEK resulted in a significant increase in the liver weight of non-pregnant rats. The absence of accompanying histopathologic lesions or increases in the activities of the serum enzymes of hepatocellular injury suggested an adaptative response, rather than a toxic effect. However, the highest combination (1000 ppm EB and 3000 ppm MEK) resulted in leakage of hepatic enzymes. In addition, the increase in liver weight was more pronounced than after individual treatments (i.e., not simple summation of the individual solvent effects), suggesting more than additive effects at these concentrations. The classical biochemical markers of kidney damage (e.g., serum creatinine and urea) were unaffected, except from slight changes in few urinary parameters (mainly increase in the urinary volume) in the high EB/MEK exposure group. The absence of gross and histopathological changes in the kidney of treatment groups indicated that EB and MEK, individually or in combination, have minimal nephrotoxic effects. These results are in accordance with previous inhalation studies. Fisher 344 rats exposed to 382 or 782 ppm EB for four weeks had increased liver weights, but normal histopathology, and urine and serum biochemistry (Cragg et al., 1989). Concentration-related increases in weights of liver and kidney, in the absence of microscopic lesions, were also observed in F344 rat exposed to 250–1000 ppm EB for 13 weeks (NTP, 1992). Male Wistar rats exposed up to 600 ppm EB for 16 weeks showed no increase in serum ALAT, nor liver cell necrosis. Nevertheless, proliferation of liver smooth endoplastic reticulum was seen, consistent with the stimulation of mixed function oxidase and other enzyme activities (Elovaara et al., 1985). Increased liver weight was also reported in rats inhaling 800 ppm MEK for four weeks (Toftgard et al., 1981) or 5000 ppm for 13 weeks (Cavender et al., 1983).

In addition to the dose level, our metabolic findings show that combined exposure to solvents can produce complex results, depending on the number of exposures. Mandelic acid results from oxidations of the side chain of EB, and is one of its major urinary metabolites following inhalation exposure in rats and humans (Ensgtro¨m et al., 1984, 1985). In our study, co-exposure with MEK was associated with changes in EB metabolism. Thus, MEK tended to reduce the urinary levels of mandelic acid in a concentration-related manner after acute or repeated co-treatments with the low concentration of EB (250 ppm), or after acute co-treatments with the high EB concentration (1000 ppm). In vitro experiments using human liver microsomes have shown that the initial step of EB metabolism is predominantly metabolised by CYP 2E1 (Sams et al., 2004). MEK is metabolized by reductive, and mostly by oxidative pathways. This latter biotransformation process probably involves the microsomal monooxygenase system (DiVincenzo et al., 1976; Dietz et al., 1981). Under these exposure patterns, it is possible that MEK may compete with EB for the same enzymatic site of metabolism. These results are consistent with previous reports on combinations of MEK and aromatic hydrocarbons. Thus, simultaneous inhalation exposure of rats to toluene (50 or 100 ppm) and MEK (200 and 400 ppm) resulted in a reduction in the urinary excretion of hippuric acid, an oxidative metabolite of toluene (Uaki et al., 1995). The oxidation of the side chain of meta-xylene, producing methyl hippuric acid, was also inhibited in the presence of MEK in rats and humans (Liira et al., 1988, 1991). In contrast to the other co-exposure regimens, repeated exposures to the highest combination of EB and MEK led to a steep

increase in the excretion of mandelic acid. EB and MEK are known to enhance liver microsomal enzyme activities, including cytochromes P450 (Elovaara et al., 1984, 1985; Brady et al., 1989; Raunio et al., 1990; Liira et al., 1991; Nedelcheva, 1996; Sequeira et al., 1992, 1994; Backes et al., 1993; Raymond and Plaa, 1995; Yuan et al., 1995, 1997; Bergeron et al., 1999; Serron et al., 2000). Probable increase in hepatic metabolic capability induced by continued and high level exposures to MEK and EB, may have modified EB metabolism and kinetics (e.g., enhanced metabolite formation). The differences in EB excretion profile suggest that metabolic, and possibly toxic, interactions may change during the course of maternal exposure. The timing of interaction may be important in regard to the critical periods of the development susceptibility.

In the current study, simple analyses were applied to examine the effects of combined EB and MEK, i.e., twoway analysis of variance and arithmetic sum of the effects of individual compounds (i.e., effect summation). More thorough analyses would be necessary to further estimate and characterize the greater-than-additive interactions observed in several cases, in particular, a more extensive dose-response evaluation would be required (Silva et al., 2002). Several approaches have been proposed to properly detect interactions of chemicals in mixture, including physiologically based toxicokinetic-pharmacodynamic modeling, response surface analysis, and response-addition (i.e., independent action) and dose-addition methods (Groten et al., 2004; Yang et al., 2004;Monosson, 2005). Several statistical designs have also been developed, such as fractional or full factorial design (Groten et al., 1996). Thus, the concentration- addition model has proved to be useful to predict the mixture effects of estrogenic or antiandrogenic chemicals (Payne et al., 2001; Silva et al., 2002; Birkhoj et al., 2004). From the literature available, only few in vivo developmental toxicity studies have used these tools to analyze mixture data, non-additive effects were reported, however (Narotsky et al., 1995; Nelson et al., 1999).

In conclusion, results from this study provide no evidence of developmental toxicity interaction of inhaled EB and MEK in rats, and suggest that the default assumption of additivity may be appropriate to estimate the maternal and developmental effects of EB and MEK following concurrent inhalation exposures at low concentration levels. However, the possibility that other treatment conditions might result in a different conclusion cannot be excluded. Thus, this study also highlights the importance of the experimental design when studying combined effects of chemicals.

References

Agency for Toxic Substances and Disease Registry, Atlanta, GA (ATSDR), 2004. Guidance manual for the assessment of joint toxic action of chemical mixtures. US Department of Health and Human Services, ATSDR, Atlanta, GA. Available from: <http://www.atsdr.cdc.gov>.

Angerer, J., Lehnert, G., 1979. Occupational chronic exposure to organic solvents. VII. Phenolic compounds – Metabolites of alkylbenzenes in man. Simultaneous exposure to ethylbenzene and xylenes. International Archives of Occupational and Environmental Health 43, 145–150.

Backes, W.L., Sequeira, D.J., Cawley, G.F., Eyer, C.S., 1993. Relationship between hydrocarbon structure and induction of P450: effects on protein levels and enzyme activities. Xenobiotica 23, 1353–1366.

Barrow, M.W., Taylor, W.J., 1969. A rapid method for detecting malformations in rat fetuses. Journal of Morphology 127, 291–306.

Bergeron, R.M., Desai, K., Serron, S.C., Cawley, G.F., Eyer, C.S., Backes, W.L., 1999. Changes in the expression of cytochrome P450s 2B1, 2B2, 2E1, and 2C11 in response to daily aromatic hydrocarbon treatment. Toxicology and Applied Pharmacology 157, 1–8.

Birkhoj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H.R., Dalgaard, M., et al., 2004. The combined antiandrogenic effects of five commonly used pesticides. Toxicology and Applied Pharmacology 201, 10–20.
Brady, J.F., Li, D., Ishizaki, H., Lee, M., Ning, S.M., Xiao, F., Yang, C.S., 1989. Induction of cytochromes P450IIE1 and P450IIB1 by secondary ketones and the role of P450IIE1 in chloroform metabolism. Toxicology and Applied Pharmacology 100, 342–349.
Cavender, F.L., Casey, H.W., Salem, H., Swenberg, J.A., Gralla, E.J., 1983. A 90-day vapor inhalation toxicity study of methyl ethyl ketone. Fundamental and Applied Toxicology 3, 264–270.
Cragg, S.T., Clarke, E.A., Miller, R.R., Terrill, J.B., Ouellette, R.E., 1989. Subchronic inhalation toxicity of ethylbenzene in mice, rats, and rabbits. Fundamental and Applied Toxicology 13, 399–408.
Deacon, M.M., Pilny, M.D., John, J.A., Schwetz, B.A., Murray, F.J., Yakel, H.O., et al., 1981. Embryo- and fetotoxicity of inhaled methyl ethyl ketone in rats. Toxicology and Applied Pharmacology 59, 620–622.
Dietz, F.K., Rodriquez-Giaxola, M., Traiger, G.J., Steel, V.J., Himmelstein, K.J., 1981. Pharmacokinetics of 2-butanol and its metabolites in the rat. Journal of Pharmacokinetics and Biopharmaceutics 9, 553–576.
DiVincenzo, G.D., Kaplan, C.J., Dedinas, J., 1976. Characterization of the metabolites of methyl n-butyl ketone, methyl iso-butyl ketone, and methyl ethyl ketone in guinea-pig serum and their clearance. Toxicology and Applied Pharmacology 36, 511–522.
Dowty, B.J., Laseter, J.L., Storer, J., 1976. Transplacental migration and accumulation in blood of volatile organic constituents. Pediatric Research 10, 696–701.
Elovaara, E., Ensgtro¨m, K., Vainio, H., 1984. Metabolism and disposition of simultaneously inhaled m-xylene and ethylbenzene in the rat. Toxicology and Applied Pharmacology 75, 466–478.
Elovaara, E., Ensgtro¨m, K., Nickels, J., Aitio, A., Vainio, H., 1985. Biochemical and morphological effects of long-term inhalation exposure of rats to ethylbenzene. Xenobiotica 15, 299–308.
Ensgtro¨m, K., 1984. Metabolism of inhaled ethylbenzene in rats. Scandinavian Journal of Work and Environmental Health 10, 83–87.
Ensgtro¨m, K., Riihima¨ki, V., Laine, A., 1984. Urinary disposition of ethylbenzene and m-xylene in man following separate and combined exposure. International Archives of Occupational and Environmental Health 54, 355–363.
Ensgtro¨m, K., Elovaara, E., Aitio, A., 1985. Metabolism of ethylbenzene in the rat during long-term intermittent inhalation exposure. Xenobiotica 15, 281–286.
Freundt, K.J., Ro¨mer, K.G., Federsel, R.J., 1989. Decrease of inhaled toluene, ethyl benzene, m-xylene, or mesitylene in rat blood after combined exposure to ethyl acetate. Bulletin of Environmental Contamination and Toxicology 42, 495–498.
Greaves, P., 2000. VIII. Digestive system 2. Liver. In: Greaves, P. (Ed.), Histopathology of Preclinical Toxicity Studies. Interpretation and Relevance in Drug Safety Evaluation, second ed. Elsevier Sciences B.V., Amsterdam, pp. 432–481.
Groten, J.P., Schoen, E.D., Feron, V.J., 1996. Use of factorial designs in combination toxicity studies. Food and Chemical Toxicology 34, 1083–1089.
Groten, J.P., Heijne, W.H.M., Stierum, R.H., Freidig, A.P., Feron, V.J., 2004. Toxicology of chemical mixtures: a challenging quest along empirical sciences. Environmental Toxicology and Pharmacology 18, 185–192.
Haghdoost, N.R., Newman, L.M., Johnson, E.M., 1997. Multiple chemical exposures: synergism vs. individual exposure levels. Reproductive Toxicology 11, 9–27.

Hardin, B.D., Bond, G.P., Sikov, M.R., Andrews, F.D., Beliles, R.P., Niemeier, R.W., 1981. Testing of selected workplace chemicals for teratogenic potential. Scandinavian Journal of Work and Environmental Health 7 (Suppl. 4), 66–75.
Imaoka, S., Funae, Y., 1991. Induction of cytochrome P450 isozymes in rat liver by methyl n-alkyl ketones and n-alkylbenzenes. Biochemical Pharmacology 42, S143–S150.
Kivisto, H., Pekari, K., Aitio, A., 1993. Analysis and stability of phenylglyoxylic and mandelic acids in the urine of styrene-exposed people. International Archives of Occupational and Environmental Health 64, 399–403.
Liira, J., Riihima¨ki, V., Ensgtro¨m, K., Pfa¨ffli, P., 1988. Coexposure of man to m-xylene and methyl ethyl ketone. Scandinavian Journal of Work and Environmental Health 14, 322–327.
Liira, J., Elovaara, E., Raunio, H., Riihima¨ki, V., Ensgtro¨m, K., 1991. Metabolic interaction and disposition of methyl ethyl ketone and mxylene in rats at single and repeated inhalation exposures. Xenobiotica 21, 53–63.
Monosson, E., 2005. Chemical mixtures: considering the evolution of toxicology and chemical assessment. Environmental Health Perspectives 113, 383–390.
Narotsky, M.G., Weller, E.A., Chinchilli, V.M., Kavlock, R.J., 1995. Nonadditive developmental toxicity in mixtures of trichloroethylene, di(2-ethylhexyl)phthalate, and heptachlor in a 5 · 5 · 5 design. Fundamental and Applied Toxicology 27, 203–216.
National Toxicology Program (NTP), 1992. Toxicity studies of ethylbenzene in F344 rats and B6C3F1 mice (inhalation studies). NIH Publication No. 92-3129. Research Triangle Park, NC, USA.
Nedelcheva, V., 1996. Interaction of styrene and ethylmethylketone in the
induction of cytochrome P450 enzymes in rat lung, kidney and liver after separate and combined inhalation exposures. Central European Journal of Public Health 4, 115–118.
Nelson, B.K., 1994. Interactions in developmental toxicology: a literature review and terminology proposal. Teratology 49, 33–71.
Nelson, B.K., Snyder, D.L., Shaw, P.B., 1999. Developmental toxicity interactions of salicylic acid and radiofrequency radiation or 2- methoxyethanol in rats. Reproductive Toxicology 13, 137–145.
Ogata, M., Sugihara, R., 1978. High performance liquid chromatographic procedure for quantitative determination of urinary phenylglyoxylic, mandelic, and hippuric acids as indices of styrene exposure. International Archives of Occupational and Environmental Health 42, 11–19.
Payne, J., Scholze, M., Kortenkamp, A., 2001. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environmental Health Perspectives 109, 391–397.
Raunio, H., Liira, J., Elovaara, E., Riihima¨ki, V., Pelkonen, O., 1990. Cytochrome P450 isozyme induction by methyl ethyl ketone and mxylene in rat liver. Toxicology and Applied Pharmacology 103, 175–179.
Raymond, P., Plaa, G.L., 1995. Ketone potentiation of haloalkaneinduced hepato- and nephrotoxicity. II: Implication of monooxygenases. Journal of Toxicology and Environmental Health 46, 317–328.
Ruehl-Fehlert, C., Kittel, B., Morawietz, G., Deslex, P., Keenan, C., Mahrt, C.R., et al., 2003. Revised guides for organ sampling and trimming in rats and mice – Part 1. Experimental and Toxicologic Pathology 55, 91–106.
Saillenfait, A.M., Gallissot, F., Morel, G., Bonnet, P., 2003. Developmental toxicities of ethylbenzene, ortho-, meta-, para-xylene and technical xylene in rats following inhalation exposure. Food and Chemical Toxicology 41, 415–429.

Salewski, E., 1964. Farbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. Naunym-SchmeidebergsArchives fur Pharmakologie und Experimentalle Pathologie 247, 367.
Sams, C., Loizou, G.D., Cocker, J., Lennard, M.S., 2004. Metabolism of ethylbenzene by human liver microsomes and recombinant human cytochrome P450s (CYP). Toxicology Letters 147, 253–260.
Schwetz, B.A., Leong, B.K.J., Gehring, P.J., 1974. Embryo- and fetotoxicity of inhaled carbon tetrachloride, 1,1-dichloroethane and methyl ethyl ketone in rats. Toxicology and Applied Pharmacology 28, 452–464.
Schwetz, B.A., Mast, T.J., Weigel, R.J., Dill, J.A., Morrissey, R.E., 1991. Developmental toxicity of inhaled methyl ethyl ketone in Swiss mice. Fundamental and Applied Toxicology 16, 742–748.
Sequeira, D.J., Eyer, C.S., Cawley, G.F., Nick, T.G., Backes, W.L., 1992. Ethylbenzene-mediated induction P450 isozymes in male and female rats. Biochemical Pharmacology 44, 1171–1182.
Sequeira, D.J., Cawley, G.F., Eyer, C.S., Backes, W.L., 1994. Temporal changes P-450 2E1 in expression with continued ethylbenzene exposure. Biochimica and Biophysica Acta 1207, 179–186.
Serron, S.C., Dwivedi, N., Backes, W.L., 2000. Ethylbenzene induces microsomal oxygen free radical generation: antibody-directed characterization of the responsible cytochrome P450 enzymes. Toxicology and Applied Pharmacology 164, 305–311.
Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from ‘‘nothing’’ – eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environmental Sciences Technology 36, 1751–1756.
Skowron, J., Miranowicz-Dzierzawska, K., Zapor, L., Golofit-Szymczak, M., Starek, A., 2001. Interactions of some organic solvents: hydrocarbons and chloroalkene. International Journal of Occupational Safety and Ergonomics 7, 35–47.
Staples, R.E., Schnell, V.L., 1964. Refinements in rapid clearing technique in the KOH-Alizarin red S method for bone. Stain Technology 39, 62–63.
Tang, W., Hemm, I., Eisenbrand, G., 2000. Estimation of human exposure to styrene and ethylbenzene. Toxicology 144, 39–50.
Toftgard, R., Nilsen, O.G., Gustafsson, J.A., 1981. Changes in rat liver microsomal cytochrome P-450 and enzymatic activities after inhalation of n-hexane, xylene, methyl ethyl ketone and methylchloroform for four weeks. Scandinavian Journal of Work and Environmental Health 7, 31–37.
Uaki, H., Kawai, T., Mizunuma, K., Moon, C.S., Zhang, Z.W., Inui, S., et al., 1995. Dose-dependent suppression of toluene metabolism by isopropyl alcohol and methyl ethyl ketone after experimental exposure of rats. Toxicology Letters 81, 229–234.
Ungvary, G., Tatrai, E., 1985. On the embryotoxic effects of benzene and its alkyl derivatives in mice, rats, and rabbits. Archives of Toxicology Supplement 8, 425–430.
Wilson, J.G., 1965. Methods for administering agents and detecting malformations in experimental animals. In: Wilson, J.G., Warkany, J. (Eds.), Teratology: Principles and Techniques. Univ. of Chicago Press, Chicago, pp. 262–277.
World Health Organization (WHO), 1996. Environmental Health Criteria 186. Ethylbenzene. WHO, Geneva. Yang, R.S.H., El-Masri, H.A., Thomas, R.S., Dobrev, I.D., Dennison Jr.,
J.E., Bae, D.S., et al., 2004. Chemical mixture toxicology: from descriptive to mechanistic, and going on to in silico toxicology. Environmental Toxicology and Pharmacology 18, 65–81.
Yuan, W., White, T.B., White, J.W., Strobel, H.W., Backes, W.L., 1995.
Relationship between hydrocarbon structure and induction of P450: effect on RNA levels. Xenobiotica 25, 9–16.

Yuan, W., Sequeira, D.J., Cawley, G.F., Eyer, C.S., Backes, W.L., 1997. Time course for the modulation of hepatic cytochrome P450 after administration of ethylbenzene and its correlation with toluene metabolism. Archives of Biochemistry and Biophysics 339, 55–63.