13 September 2012

Food Standards Australia New Zealand
PO Box 7186
CANBERRA BC ACT 2610

Via e-mail: submissions@foodstandards.gov.au

Dear FSANZ,

Submission – Public Comment on A1043 World Health Organisation (WHO) Limits for Packaged Water

The Australian Beverages Council (the Beverages Council) is the peak body representing the $7 billion non-alcoholic beverage industry. Membership of the Beverages Council comprises over 95% of the non-alcoholic industry's production volume, and is comprised of multi-national companies and small and medium businesses, and includes membership of the Australasian Bottled Water Institute (ABWI).

The areas that are proposed for amendment by FSANZ, in relation to the WHO Limits proposed by ABWI, include fluoride level and styrene. The Beverages Council has consulted its membership most likely to be impacted by these proposed changes and submit that this limit provides a workable level for fluoride so as to ensure first and foremost safety for consumers with little impact on domestic supply.

To that end, the Beverages Council can support the decrease to fluoride levels permitted in bottled water.

However, **Beverages Council and ABWI do not support the proposed increase to levels of styrene** permissions for in bottled water. The proposed increase from 0.02 mg/L to 0.03 mg/L is to the maximal level permitted by the Australian Drinking Water Guidelines (2011).

The Australian Drinking Water Guidelines provide detailed information surrounding the origins of styrene in the food supply. The source of styrene in water described in the Australian Drinking Water Guidelines is industrial waste, with major sources of styrene being discharge from rubber and plastic factories; and leaching from landfills. These sources are contaminants rather than processing aids.

Australian Beverages Council Ltd
Level 1, 6-8 Crewe Place Rosebery NSW 2018 Australia • Ph: +61 2 9662 2844 • Fax: +61 2 9662 2899
Email: info@australianbeverages.org • www.australianbeverages.org

We have recently confirmed with ion exchange resin suppliers (Pheta Industries, pers comm) and filtration suppliers (Pall Australia, pers comm) that increases in styrene levels are not associated with the processing aids they supply. We have also discussed with packaging consultant, Edward Kosior of Nextek (Nextek, pers comm) who confirmed that polycarbonate, polyethylene terephthalate (PET), polycarbonate and glass are commonly used bottled water packaging. These do not contribute any significant amount of styrene into bottled water.

Detectable Limits By Consumers

Styrene has a sweet odour and odour thresholds for solutions in water range from 0.02 to 2.6 mg/litre (van Apeldoorn et al, 1985). At 60 °C, a lower odour threshold of 0.0036 mg/litre has been reported (Alexander et al, 1982).

An odour taint in bottled water is undesirable particularly if the odour is associated with a chemical that is a group 2B substance that has possible carcinogenicity to humans (Ahmad, 2007). Such an odour taint is sufficient to result in a product recall as recently experienced by a large manufacturer of bottled water in England, who as a result has been faced with undesirable financial consequences.

Styrene Used As Food Packaging.

Styrene is used in some food packaging although there is no history of use in manufacture of beverages in Australia. Examples of packaging containing styrene include egg cartons, foam meat trays, clear take away containers, instant noodles, plastic cutlery, toys, cups and CD cases.

Scientific Literature – Human Exposure

In a paper from Germany, Tang et al (2000) estimated human exposure to styrene and ethylbenzene. Daily styrene exposure totalled an annual exposure of 6.7 to 20.2 mg/person, and reported levels of up to 0.005 mg/L styrene detected in food that originated in packaging. Tang asserts that most food sources do not contain any styrene at all and the only source could be packaging. According to Tang, the other of the main sources of styrene appears to be environmental, with average annual respiratory intake being

6.5mg/person, or 90% of the total intake suggested in this Dutch study. This determination excludes impacts of cigarette smoking.

Scientific Literature - Toxicology

An investigation by Date et al (2002) found styrene had no apparent estrogenic, androgenic, anti-androgenic or thyroid activity up to concentrations of 10^{-5} mol/L (~1.0 mg/L) in short term rat studies, however, endocrine disruption was not ruled out nor was longer term effects on human health.

According to the International Agency for Research on Cancer, at last review in 1994 styrene was considered as possibly carcinogenic. With limited evidence from human and animal studies, the category of 2B was maintained.

Scientific Literature – Leaching Studies

Foam cups, often used for hot drinks, are reported as a possible source of styrene in beverages and are discussed by Ahmad & Bajahlam (2007). It was investigated by these researchers that leaching of styrene and other aromatic compounds was found in drinking water from polystyrene bottles in countries other than Australia. The maximum level of styrene found in hot liquids stored in styrene reached 0.0295 mg/L, and increased to 0.06953 mg/L after one year storage.

Recommendation

As bottled water is sourced from ground water and in some cases, processed potable supply, we suggest **the original level of 0.02 mg/L is appropriate**. This will adopt a globally harmonised standard that is safe and acceptable for consumers, and achievable by Australian and New Zealand manufacturers.

We thank FSANZ for the opportunity to provide this submission at public comment phase. [redacted]

Yours sincerely,

[redacted]

Health and Regulatory Affairs Manager

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Leaching of styrene and other aromatic compounds in drinking water from PS bottles

Maqbool Ahmad*, Ahmad S. Bajahlan

Environmental Control Department, Directorate General for Royal Commission at Yanbu, P.O.Box 30031 Yanbu Al-Sinaiyah, Kingdom of Saudi Arabia, E-mail: bajahlan@rc-ynb.com

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Abstract

Bottled water may not be safer, or healthier, than tap water. The present studies have proved that styrene and some other aromatic compounds leach continuously from polystyrene (PS) bottles used locally for packaging. Water sapmles in contact with PS were extracted by a preconcentration technique called as "purge and trap" and analysed by gas chromatograph-mass spectrometer (GC/MS). Eleven aromatic compounds were identified in these studies. Maximum concentration of styrene in PS bottles was 29.5 μg/L. Apart from styrene, ethyl benzene, toluene and benzene were also quantified but their concentrations were much less than WHO guide line values. All other compounds were in traces. Quality of plastic and storage time were the major factor in leaching of styrene. Concentration of styrene was increased to 69.53 μg/L after one-year storage. In Styrofoam and PS cups studies, hot water was found to be contaminated with styrene and other aromatic compounds. It was observed that temperature played a major role in the leaching of styrene monomer from Styrofoam cups. Paper cups were found to be safe for hot drinks.

Key words: styrene; polystyrene (PS); ethyl benzene; leaching; styrofoam

Introduction

Bottled water is now so popular that there are more than 700 brands of water worldwide (Gordon, 2001). Almost all type of plastics is used for bottling purposes. Even polystyrene (PS) is also used for small packaging. An estimate is that 1.5 million tones of plastic is used yearly by the bottled water industry. Buying bottled water is not a long-term sustainable solution to have access to healthy water. The plasticizer start migration from day one, destruction processes, aging of plastics and the presence of unbound low-molecular-mass compounds considerably increase migration level. The extent to which migration occurs depending upon such factors as the contact area, type of plastic, temperature, and contact time. The U.S. Food and Drug Administration (FDA) call these plastics "Food Contact Substances" (FDA, 2004).

Rigid PS and PS-related plastics, which are used as food packaging materials, have longer history of use than poly vinyl chloride (PVC). Some of the physical characteristics of PS, for example, its low impact strength and chemical resistance, have led to the development of other food grade plastics in which styrene is co-polymerized with monomers like butadiene and acrylonitrile to give it more flexibility. Styrofoam is cross-linked PS, which is blown out using blowing agent during manufacture. The Styrofoam that keeps coffee hot and eggs from breaking is an economical product used in everything from packing peanuts to sanitary ware. It is used not only in the rigid molded state as food containers (dairy products) but is now manufactured in a form suitable for use as reusable cutlery and in the foamed state, as drink containers (for hot drinks).

The fact that styrene can adversely effect humans in a number of ways raises serious public health and safety questions regarding its build-up in human tissue and the root cause of this build-up. Long-term exposure to small quantities of styrene can cause neurotoxic (fatigue, nervousness, sleeplessness), hematological (low platelet and hemoglobin values), cytogenetic (chromosomal and lymphatic abnormalities), and carcinogenic effects (Dowty *et al*., 1976). In 1987, the International Agency for Research on Cancer (IARC), re-classified styrene from a Groups 3 (not classifiable as to its carcinogenicity) to a Group 2B substance (possibly carcinogenic to humans) (ATSDR, 1992). There are evidences that styrene is a carcinogen and neurotoxin (Dowty *et al*., 1976), and it has also been linked to reproductive problems. Styrene is also found in the milk of feeding females (Brown, 1991). Women exposed to low concentrations of styrene vapors in the workplace are known to have a variety of neurotoxic and menstrual problems. Certain styrene oligimers have proliferative activity on MFC-7 human breast tumor cells and binding affinity for human estrogen receptor α (Ohyama *et al*., 2001). Styrene is mainly biotransformed to styrene-7,8-oxide via the mixed function oxidase system. It has been reported

*Corresponding author. E-mail: maqbool_60@yahoo.com.

(Van Duuren, 1969; Cohen *et al*., 2002) that styrene-7,8-oxide bind with DNA and induces tumors and probably causes carcinogenicity.

Styrene is a contaminant in all PS foam packages. It was first documented in 1972 (Figge, 1972) and then again in 1976 (Dowty *et al*., 1976). Styrofoam leach styrene into the liquids they contain. Leaching of styrene increased by heating (Withey, 1976). Styrene has been found in food, packed in PS containers (FDA, 2003; Health Canada, 1993). Styrene has been reported in yogurt, cream, salads, soft cheese, margarines, hot or cold beverage, fresh and cooked meat, candied fruit and fast food packed in PS (Miller *et al*., 1994). Styrene level has also been measured in human tissue. According to a study (ASTDR, 1992) styrene was detected in adipose tissues, and blood.

Considering the importance of toxic effect of styrene and its leachability in food materials and water from PS, the present studies were conducted. Water samples packed in PS bottles and styrofoam, PS and paper cups were collected from local market of Yanbu Industrial City (Saudi Arabia). Apart from the packing and date of expiry no other record of these bottled water was available. All these samples were assayed for styrene monomer contents.

1 Materials and methods

1.1 Sample collection and preservation

All the water samples in PS bottles were collected from local market and were analyzed on the same day. PS disposable cups, paper cups and styrofoam cups were also purchased from local market: PS bottles filled with water (250-ml); styrofoam cups (250-ml); PS disposable cups (80-ml); paper cups (250-ml). Before analysis pH of the each sample was also measured and it was: bottled water 7.2 to 7.5; potable water 7.9 to 8.1.

1.2 Analytical techniques

Modern chromatographic techniques including capillary gas chromatography and high-performance liquid chromatography (HPLC) together with highly selective detectors like flame ionization (FID), electron capture (ECD), nitrogen-phosphorous (NPD), mass spectrometer (MS), ultraviolet (UV), electrochemical and fluorescent, ensure separation, identification, and precise determination of the majority of toxic substances migrating from plastics to contact media at the levels required for safety evaluation. Usually sample preparation is performed by a number of different analytical techniques, including purge and trap (P&T) (Munch, 1995), liquid-liquid extraction (LLE), solid phase extraction (SPE), headspace (Withey, 1976) and direct aqueous injection in combination with a chromatographic system. In the present study samples were extracted and preconcentrated on VOCARB® 3000 trap by Tekmar Velocity XPT accelerated purge and trap sample concentrator and analyzed by Varian 3800 Gas chromatograph and Saturn 2200 mass spectrometer (GC/MS). USEPA method 524.2 (Munch, 1995) was followed as guideline for extraction.

1.3 Operating conditions for purge and trap and GC/MS

Optimized operating conditions for purge and trap are given below: transfer line temperature 150°C; desorption temperature 250°C; purge time 11 min; desorption time 2.0 min; purge temperature ambient; desorption flow 300 ml/min; purge flow 40 ml/min; bake temperature 300°C.

Detailed condition for GC temperature programming, column, and MS parameters are given below: injector temperature 200°C; carrier gas (helium) 99.9999%; carrier gas flow: 1.0 ml/min; transfer line temperature 250°C; emission current 20 μA; ionization mode: EI; column: FactorFour Capillary column VF-5MS 30 m × 0.25 mm ID, df=0.25 Varian; column temperature 35°C (3 min) to 150°C (1.0 min) at 12.5 °C/min then to 280°C (2 min) at 30°C/min.

1.4 Reagents

Reagent water: reagent water was generated from Millipore Milli Q ultra pure water purification unit; stock solutions: stock standard solutions of 1000 mg/L in methanol from Supelco; sub stock solution: 200 mg/L in methanol; working standards: 2.5, 10, and 20 mg/L in methanol.

Velocity XPT purge and trap was connected with gas chromatograph, calibrated the P&T-GC system using high purity nitrogen as purging gas. Each calibration standard was extracted and preconcentrated by directly injecting (2.0 μl of working standard mixture in the syringe containing 5 ml reagent water) in the sparger under the optimized condition and analyzed by GC/MS. For quantification purposes qualifier ion and retention time (RT) for each compound were used. For benzene, toluene, ethyl benzene and styrene qualifier ions were 78, 91, 91 and 104 and RT 3.549, 5.352, 7.030 and 7.556 min respectively. Reagent water (5.0 ml) spiked with 2 μl working standards (2.5, 10.0 and 20.0 mg/L) was equivalent to 1.0, 4.0, and 8.0 μg/L of each compound respectively.

Concentration of individual compound was determined by peak area using software MS Workstation Version 6.4. Response factors (RF) for each compound was calculated and three point calibration curves were prepared.

1.5 Experimental method

The details of different experiments are as follows: (1) bottled water samples were analyzed within 4 h after collection; (2) in styrofoam and paper cups studies, 200 ml boiling water was added in the cups and kept at ambient temperature for 60 min; (3) in PS disposable cups, 70 ml boiling water was added and kept at ambient temperature for 60 min.

After optimizing GC/MS and purge and trap conditions and preparing calibration curves, 5 ml water sample was carefully sucked into the syringe and injected into sparger. High purity nitrogen (99.999%) was purged at 40 ml/min for 11 min. Purged volatiles were preconcentrated on VOCARB® 3000 trap. Then dry air was purged to remove moisture. Trap temperature was increased to 250°C and

adsorbed volatiles were desorbed and transferred to GC injector through a preheated transfer line with high purity (99.9999%) helium gas. Analysis was performed by GC/MS and concentration of individual component was calculated by calibration curve. Reference spectra of all the identified compounds produced by GC/MS system were recorded.

2 Results and discussion

2.1 Bottled water

The main work was to assess the extent to which styrene is leached in water from PS material. In this study water in PS bottles was found to contain styrene and some other aromatic compounds (Table 1). Results show that styrene was significantly high in all PS bottled water. Locally three brands of PS bottled water were available and all were found contaminated with styrene, toluene, ethyl benzene and benzene. Maximum concentration was of styrene (Table 1). Concentration of other identified aromatic compounds was much less than styrene.

Presence of styrene in water is supposed to be due to the leaching of free styrene monomer present in the PS plastic. As it has been reported that styrene monomer present in PS ranges from 300 to 1000 mg/kg (EU, 2002). The FDA requires that residual styrene monomer level in basic styrene polymer products intended for fatty food should not be more than 0.5% (5000 mg/kg) (FDA, 2004). The 2nd major compound found in water stored in PS was ethyl benzene. Its concentration ranged from 0.42 to 3.21 μg/L. As it is known that styrene is manufactured from ethylene benzene and traces of ethyl benzene are always there. Impurities vary by plant and production method and can include ethyl benzene (<0.1%), isopropyl benzene, toluene, benzene, *p*-xylene, 2-phenyl propene etc. (EU, 2002). Presence of ethyl benzene, toluene and benzene indicates that these impurities were present in styrene used for the production of PS. These impurities also leached along with styrene in the water. Presence of styrene in food material and water packed in PS containers is also reported in literature (EU, 2002; Withey, 1976; FDA, 2003; Paul, 2003). Guideline value by WHO (2004) and maximum permissible limits by Royal Commission (RC) for Jubail and Yanbu, Saudi Arabia (RC, 2004) are given in Table 2. Maximum concentration of styrene in this study was 29.5 μg/L, higher than World Health Organization (WHO) guide line values and RC maximum permissible limits (20 μg/L).

Table 2 WHO/RC maximum permissible limits (μg/L)

Compound name	WHO limits	RC limits
Benzene	10	10
Ethyl benzene	300	300
Toluene	700	700
Styrene	20	20

Correlation of results with packing date labeled on the bottle (Table 1) indicates that storage time have significant effect on leaching of monomers. Further studies were conducted to investigate the effect of storage time. Three bottles of the same brand with same packing date and batch number were purchased from the market. Packing date on all the three bottles was April 2005, the first analysis was done on 9 June 2005. Every 2nd month analysis was repeated. As the expiry period mentioned on bottles was one year, hence last analysis was completed in February 2006. For analysis, aluminum cover of each bottle was punctured with a sharp needle and 5 ml water was sucked with syringe for analysis. Bottles were again sealed with paper tape and left under ambient atmospheric conditions. Detailed results are given in Table 3 and average analyses of three bottles are plotted in Fig.1. Results indicate that there is significant increase of styrene with the passage of time. Increase in styrene and ethyl benzene was almost in the same ratio. Concentration of styrene was almost three times higher than WHO guide line values at the end of experimental period. There was no significant increase in benzene and toluene concentration. Apart from styrene and the other three quantified compounds, propyl benzene, 1-ethyl 4-methyl benzene, α-methyl styrene, (1-methylethyl) benzene, 2-propenyl benzene, 1-ethyl 3-methyl benzene and *p*-xylene were also identified in some of the experiments, where the concentration of styrene was significantly higher. Presence of these compounds indicates that traces of these compounds were present as impurities of styrene

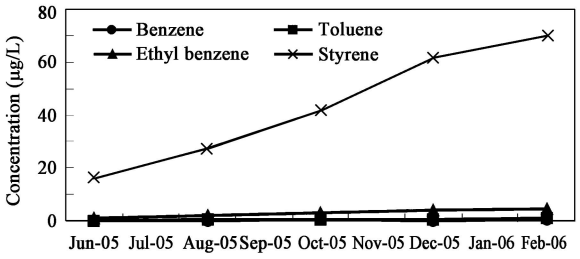

Fig. 1 Effect of storage time on leaching of styrene from PS bottles.

Table 1 Leaching of aromatic compounds in water from PS bottles (μg/L)

Brand	Packing date	Date of analysis	Benzene	Toluene	Ethyl benzene	Styrene	Total
Brand 1	October 04	31/1/05	0.16	0.14	1.58	20.35	22.22
Brand 1	October 04	31/1/05	0.22	0.18	1.20	20.05	21.64
Brand 1	May 05	9/7/05	0.13	0.21	1.77	16.00	18.11
Brand 2	April 04	31/1/05	0.80	0.41	0.76	16.28	18.24
Brand 2	May 04	9/7/05	0.13	0.21	1.77	20.05	22.16
Brand 2	April 05	12/7/05	0.13	0.20	1.60	15.84	17.77
Brand 3	June 04	31/1/05	0.16	0.19	3.21	29.50	33.06
Brand 3	October 04	31/1/05	0.73	0.28	2.46	26.96	30.42
Brand 3	June 05	9/7/05	0.16	0.04	0.42	12.29	12.90

Table 3 Effect of storage time on leaching of aromatic compounds from PS (μg/L)

Bottle	Date of analysis	Benzene	Toluene	Ethyl benzene	Styrene	Total
Bottle 1	6/9/2005	0.13	0.20	1.60	15.84	17.77
Bottle 2	6/9/2005	0.18	0.251	0.85	17.28	18.56
Bottle 3	6/9/2005	0.14	0.28	1.08	15.45	16.95
Bottle 1	8/9/2005	0.25	0.45	2.15	25.87	28.72
Bottle 2	8/9/2005	0.18	0.51	1.95	27.45	30.09
Bottle 3	8/9/2005	0.31	0.39	2.01	28.56	31.27
Bottle 1	10/7/2005	0.25	0.44	3.15	42.25	46.09
Bottle 2	10/7/2005	0.41	0.39	3.08	40.42	44.30
Bottle 3	10/7/2005	0.38	0.64	2.98	42.25	46.25
Bottle 1	12/8/2005	0.28	0.45	4.29	59.25	64.27
Bottle 2	12/8/2005	0.24	0.68	4.15	62.31	67.38
Bottle 3	12/8/2005	0.19	0.71	4.07	64.15	69.12
Bottle 1	2/8/2006	0.28	1.24	4.54	69.25	75.31
Bottle 2	2/8/2006	0.26	0.78	4.68	68.14	73.86
Bottle 3	2/8/2006	0.41	0.61	5.06	71.21	77.29

in PS or by aging of PS, styrene may be degraded in to these by-products.

2.2 Styrofoam cups

Styrofoam cups, which had been examined for styrene monomer content, are used locally for tea and coffee. Hence it is very important to study the leaching capacity of these materials. In this study each Styrofoam cup (250-ml) was filled with 200 ml of either hot or cold water, capped or left open and allowed to stand for 60 min. The water was then analyzed for styrene monomer and other aromatic compounds. These cups are generally used for hot drinks, hence it was also important to investigate the effect of temperature on styrene leaching. For this study Styrofoam cups were filled with 200 ml potable water at 50, 70, 90 and 100°C and covered properly. After 60 min water from each cup was analyzed. The results (Table 4) show that at 50°C there was no leaching of any chemical in water. At 70°C only traces of styrene and ethyl benzene were present (1.16 and 0.57 μg/L). At 90°C almost all the four components were identified and their total concentration was increased to 11.49 μg/L. The same increasing trend was noted at 100°C as it is clearly shown in chromatograms (Fig.2). Total concentration of all the four compounds was increased to 21.68 μg/L at 100°C (Fig.3). These results indicate that temperature plays a major role in the leaching of styrene and other aromatic compounds.

Different experiments were conducted to study the leaching of styrene and other aromatic compounds. In all these experiments boiling water was added in the Styrofoam cups and kept at ambient temperature for 60

Table 4 Temperature effect on leaching of aromatic compounds in Styrofoam cups (μg/L)

Temperature (°C)	Benzene	Toluene	Ethyl benzene	Styrene	Total
Ambient	0.00	0.00	0.00	0.00	0.00
50	0.00	0.00	0.00	0.00	0.00
70	0.00	0.00	0.57	1.16	1.73
90	0.06	0.13	1.95	9.35	11.49
100	0.06	0.21	4.05	17.37	21.68

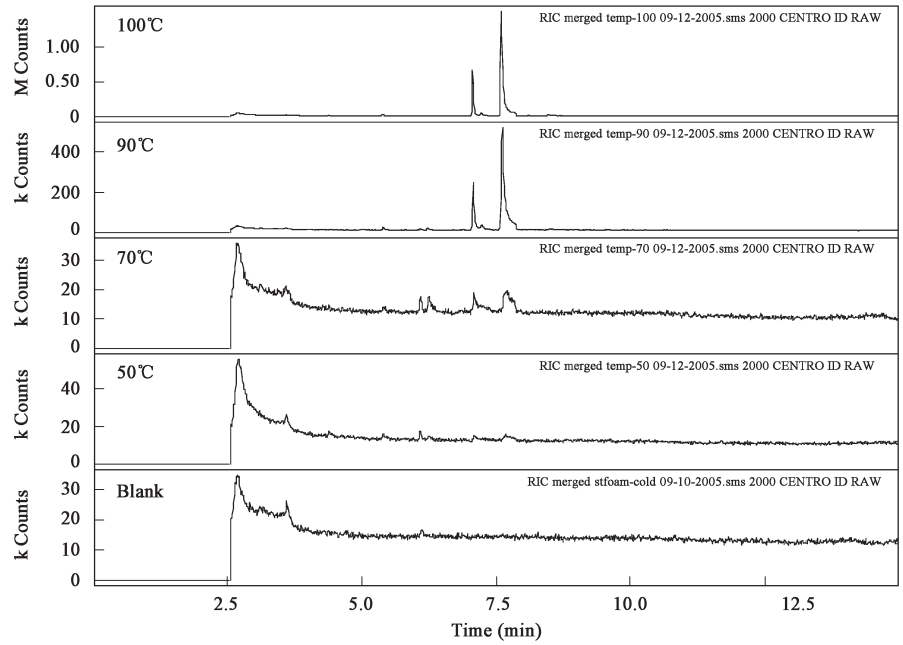

Fig. 2 Chromatograms showing the effect of temperature.

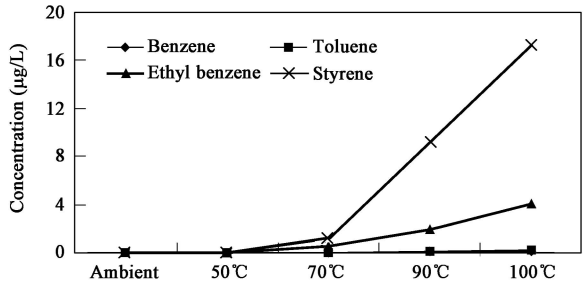

Fig. 3 Effect of temperature on leaching of styrene from styrofoam cups.

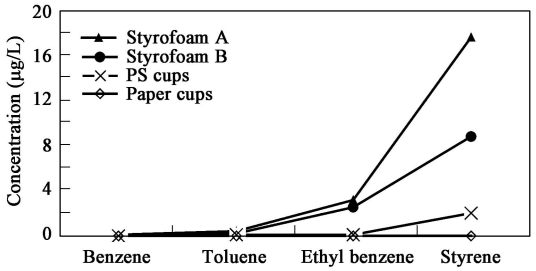

Fig. 5 Leaching of aromatic compounds from different materials.

min. The purpose was to maintain the same atmospheric conditions in which these cups are used in daily life. There was a significant difference in the results from the cups kept open and closed (Fig.4). Obviously it is clear that from hot water in open cups, styrene and other aromatic compounds easily escaped from the surface due to high vapor pressure and low solubility in water. It is also reported that styrene released to surface water is rapidly lost through volatilization (EU, 2002). In closed cup volatilization loses were much less, hence styrene and other aromatic compounds concentration was higher. Similarly contents of styrene monomers also varied in cups from different manufacturer (Table 5, Fig.5). This may be due to the monomer contents present in PS. In some of the results concentration of styrene was much higher than WHO guideline value (Table 2). Concentration of other identified aromatic compounds was always less than WHO limits. Apart from above quantified four compounds the other identified compounds were: propyl benzene, 1-ethyl 4-methyl benzene, α-methyl styrene, (1-methylethyl) benzene, 2-propenyl benzene, 1-ethyl 3-methyl benzene and *p*-xylene.

The quality of styrofoam cups is also important in leaching of styrene monomer. During these studies Styrofoam cups from two different manufacturer were purchased from super market and were labeled as "A" and "B". Results show that (Table 5) on average in "A" the styrene concentration (17.69 μg/L) was much higher than in "B" (8.85 μg/L). The concentration of other three compounds

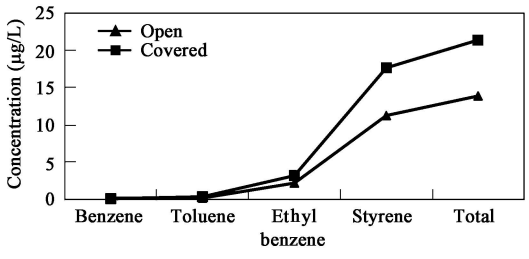

Fig. 4 Styrene leaching from open and covered styrofoam cups.

Table 5 Leaching of aromatic compounds from different materials (μg/L)

Cups type	Benzene	Toluene	Ethyl benzene	Styrene	Total
Styrofoam A	0.13	0.38	3.21	17.69	21.41
Styrofoam B	0.00	0.17	2.55	8.85	11.57
PS cups	0.00	0.06	0.13	2.03	2.21
Paper cups	0.00	0.00	0.00	0.00	0.00

was also slightly higher in "A". This may also be due to manufacturing defect and polymerization efficiency of the polymer. In "A" the unbound monomer may be more than in "B" and the same have been leached in the water. It may also be due to the aging of the styrofoam, storage conditions and manufacturing process. As these Styrofoam cups were purchased from local market, hence it can only be concluded that in "A" unbound monomer were higher than in "B". The same has been reported in literature that styrene monomer contents vary in different brands of PS (EU, 2002).

Small PS cups (80 ml capacity) locally used for tea and coffee were also studied for leaching of styrene monomer. In these cups 70 ml boiling water was added and analyzed after 60 min. From PS cups styrene monomers also migrated in hot water. The concentration of all the identified components was much less than in styrofoam. Paper cups (250 ml capacity) were also used in these studies. Both hot and cold water (200 ml) was added in paper cups and analyzed after 60 min. Even traces of organic volatile compounds were not detected in both hot and cold water. This indicates that paper cups are safe for hot drinks. These results show that leaching capacity of styrofoam cups is much higher than PS cups. In Styrofoam cups styrene leached more than WHO maximum allowable limit.

In almost all type of PS material, styrene monomer and some related aromatic compounds were leached in water. It may not have immediate effect but chronic effects may be observed as a result of repeated ingestion of a number of small doses, each in itself insufficient to cause an immediate acute reaction but in the long term having a cumulative toxic effect. Other three aromatics compounds are also highly toxic, but their concentration is much less than WHO guideline values. Chances are there that styrene might have synergetic effect with other aromatic compounds as it has been stated that in combination with other commonly used products, the toxicity of the migratory chemicals from plastic can be potentiated by synergy (Paul, 2003). Thus this and other similar chemicals have introduced a problem of protracted action of low concentrations upon human health. It is true that plastic ingredients do not act like pesticides (or a variety of other highly bioactive substances), and one can hardly expect immediate and pronounced clinical manifestations of their toxic action. The occurrence of acute toxicity due to plastic used in contact with food and drinking water is most unlikely, since only trace quantities of toxic substances are likely to migrate. However, it would be a great un-

derestimation to consider plastic ingredients (indirect food additives) as presenting no real public health threat. The timing of the exposure can be much more relevant than its dose. Most vulnerable times are in periods of rapid growth, such as those in embryo and children right up to puberty (Paul, 2003).

3 Conclusions

Considering the toxic characteristic of styrene and leaching in water and other products, PS material should be avoided for food packaging. Especially PS rigid and foam cups should not be used for hot drinks. Paper cups are safe for hot drinks and have no threat as from PS cups. It is also recommended that public awareness program shall be launched to avoid Styrofoam cups for hot drinks.

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Research Section

Endocrine-disrupting effects of styrene oligomers that migrated from polystyrene containers into food

K. Date*, K. Ohno, Y. Azuma, S. Hirano, K. Kobayashi, T. Sakurai, Y. Nobuhara, T. Yamada

Central Research Institute, Nissin Food Products Co., Ltd, 2247, Noji-Cho, Kusatsu, Shiga 525-0055, Japan

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Abstract

The endocrine-disrupting effects of styrene dimers (SD: NSD-01, -08 and -09) and styrene trimers (ST: NST -01, -03 and -12), which migrated from polystyrene (PS) containers into instant food, were investigated together with styrene monomer (SM) using in vitro and in vivo assays. In the estrogen (ER) and androgen receptor (AR) binding assay, SM, SD and ST showed no binding activity at concentration of 10^{-10}–10^{-5} mol/l. In order to evaluate the estrogenic activity in vivo, the uterotrophic assay was conducted. When prepubertal and ovariectomized adult rats were dosed with SM, SD and ST for 3 days by subcutaneous injection, these compounds did not induce significant increase in uterine weight. Additionally, to evaluate anti-androgen activity in vivo, the Hershberger assay for anti-androgenic activity in the presence of testosterone treatment was conducted. When castrated, testosterone-treated immature male rats were dosed SM, SD and ST for 7 days by oral gavage, these compounds did not induce a decrease in the seminal vesicle, ventral prostate and levator ani plus bulbocavernosus muscle weights. To evaluate the effects on hormones other than sex hormones, the thyroid hormone receptor (TR) binding assay and rat serum prolactin (PRL) was conducted. In the TR binding assay, SM, SD and ST showed no binding activity at a concentration of 10^{-5} mol/l. When ovariectomized rats were dosed with SM, SD and ST for 3 days by sc injection, the results showed there was no change in rat serum PRL. From the above these results, we concluded that SM, SD and ST exhibit no apparent estrogenic, androgenic, anti-androgenic and thyroid activity.

Keywords: Instant noodle cup; Polystyrene container; Styrene monomer; Styrene dimer; Styrene trimer; Estrogen receptor; Androgen receptor; Thyroid hormone receptor; Uterotrophic assay; Hershberger assay; Serum prolactin

1. Introduction

In modern societies, we live in an environment that contains huge quantities of chemicals that contribute to our progress and prosperity, and have benefited mankind enormously. On the other hand, the safety of these chemicals, particularly the safety and hygiene of the containers and packaging used in contact with food, has become a controversial social issue. In Japan, materials used to package food are regulated by the Food Hygiene Law that specifies standards. For polystyrene (PS) cups used in a container for instant noodles, the total volatile components contained in the material when hot water is employed is regulated to 2000 ppm or less and styrene monomer is specified to 1000 ppm or less. All products currently marketed have conformed to these specifications.

In 1991, although scientific data had not been published, Colborn et al. (1996) described styrene dimer (SD) and styrene trimer (ST) as an endocrine-disrupting chemical in the Wingspread declaration. Following this, SD and ST were described as endocrine-disrupting chemicals in a report published by an exogenous endocrine-disrupting chemical problem research group in the Ministry of Environment Agency, Japan (1998). Additionally, Kawamura et al. (1998a,b,c,d) reported that

Abbreviations: AR, androgen receptor; *p,p′*-DDE, 2-bis(4-chlorophenyl)-1,1-dichloroethylene; DES, diethylstilbestrol; DHT, 5α-dihydroteststerone; E_2, 17β-estradiol; ER, estrogen receptor; HAP, hydroxylapatite; PRL, prolactin; PS, polystyrene; SD, styrene dimer; SM, styrene monomer; ST, styrene trimer; TP, testosterone propionate; TR, thyroid hormone receptor; T_3, triiodothyronine; T_4, thyroxine

* Corresponding author. Tel.: +81-77-561-9120; fax: +81-77-561-9199.

E-mail address: k-date@mb1.nissinfoods.co.jp (K. Date).

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SD and ST remained in PS resin, and migrated from PS containers into instant noodles. Concerning the endocrine-disrupting effect of styrene, it was reported that serum prolactin level is increased by exposure to a highly concentrated styrene monomer in humans (Mutti et al., 1984; Arfini et al., 1987). In the uterotrophic assay, it was reported that 100 ppm styrene oligomers showed the same level of estrogenic activity as 5 ppb diethylstilbestrol (DES), indicating that these compounds are 20,000-fold or less than DES (Prinsen et al., 1996). On the other hand, reproductive developmental toxicity of styrene monomer showed no toxic effects (Beliles et al., 1985). Actually, the uterotrophic assay and reproductive developmental toxicity using styrene oligomers extracted from PS resin showed there were no effects on estrogenic activity (Bachmann et al., 1998; Fail et al., 1998) or reproductive functions and development (Nagao et al., 2000). As can be seen above, there are few reports on the endocrine-disrupting effects of styrene.

Our previous study of material tests and dissolution tests of PS resin or containers showed that SD and ST exist in the resin and migrate slightly into the instant noodles (Yamada, 1999; Yamada et al., 2000a,b), that had no sex hormone-like activity (Nobuhara et al., 1999; Azuma et al., 2000). In this study, the endocrine-disrupting effects of SD and ST were evaluated by in vitro assays (ER, AR and TR binding assay) and in vivo assays (uterotrophic assay using prepubertal and ovariectomized adult rats, the Hershberger assay, and rat serum PRL).

2. Materials and methods

2.1. Test compounds

Three samples of SD (NSD-01,-08 and-09) and three samples of ST (NST-01,-03 and-12) eluted from PS containers and migrated into instant noodles, were synthesized by the Central Research Institute of Nissin Food Products Co., Ltd. Their chemical structures were determined by nuclear magnetic resonance, mass spectrometry and element analysis (Fig. 1). NST-01 was a racemic mixture. NST-03 and NST-12 were mixtures of diastereomer. Their purity was determined as 98.5% or higher by gas chromatography with ionization detection (GC-FID).

2.2. Chemicals

Styrene monomer (SM; purity 99%) (Fig. 1), bisphenol A and *p*-nonylphenol were purchased from Katayama Chemical Inc. (Osaka, Japan). 17β-estradiol (E_2; purity 98%), diethylstilbestrol (DES; purity 99%), flutamide, cyproterone acetate (purity 98%), thyroxine (T_4) and triiodothyronine (T_3) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 5α-Dihydrotestosterone (DHT; purity 95%) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene (*p*,*p*′-DDE; purity 99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Testosterone propionate (TP) and corn oil were purchased from NACALAI TESQUE Inc. (Kyoto, Japan). [2,4,6,7-^{3}H(N)]estradiol ([^{3}H]E_2; 72 Ci/mmol), [17α-methyl-^{3}H]methyltrienolone ([^{3}H]R1881; 86 Ci/mmol), methyltrienolone (R1881) and L-[^{125}I]thyroxine ([^{125}I]T_4; 1250 μCi/μg) were purchased from NEN™ Life Science Products, Inc. (Boston, MA, USA). Rat prolactin (rPRL) EIA system was purchased from Amersham International plc (Buckinghamshire, UK). All other chemicals used were of reagent grade.

2.3. Animals

Crj: CD (SD) IGS rats and F344/DuCrj (Fischer) rats were obtained from Charles River Laboratories (Hino, Japan), and maintained under controlled room temperature (23.5±2 °C), humidity (55±10%), light (12 h light/12 h dark) conditions with Charles River Certified rodent diet (CRF-1) and water available ad lib. These rats were selected for the test on the basis of body weight and freedom from clinical signs of disease or injury during the quarantine period. For uterotrophic, Hershberger and serum prolactin assays, the selected rats were placed into treatment groups such that the mean body weight±S.D. for all groups was comparable on the starting day of dosing.

2.4. Estrogen receptor binding assay

The protocol for the ER binding assay was based on that described by EDSTAC (US EPA, 1998). Female 8-week-old SD rats (180–210 g) were ovariectomized. After 2 days, rats were sacrificed. Uteri were excised and homogenized with a Polytron homogenizer in ice-cold TEDG buffer [10 mmol/l Tris–HCl, 1.5 mmol/l EDTA, 10% glycerol, 1 mmol/l DTT (pH 7.4)] at 5 ml/g tissue. The homogenate was centrifuged for 60 min at 105,000 ***g*** and 4 °C. The supernatant contained low-salt unoccupied cytosolic receptors (ER-rich cytosol).

25 μl of ER-rich cytosol was incubated with 2 nmol/l [^{3}H]E_2 and 100 μl of TEDG buffer containing with test compounds or E_2 in glass tube for 18 h at 4 °C. Test compounds were dissolved in dimethyl sulfoxide (DMSO, final concentration 0.2%) and added to TEDG buffer. After the incubation period, 0.5 ml of hydroxylapatite (HAP) slurry (made in 50 mmol/l Tris–HCl, pH 7.4) was added to each tube to separate the bound [^{3}H]E_2 from the free ligand. These tubes were incubated in an ice-cold water-bath for 20 min with mixing at 5-min intervals. Tubes were centrifuged for 3 min at

600 ***g*** and 4 °C. The HAP precipitation was washed three times with 2 ml Tris buffer (50 mmol/l, pH 7.4). After washing, ice-cold ethanol was added to each tube to extract the radiolabeled E_2, and tubes were mixed at 5-min intervals. Tubes were centrifuged for 10 min at 600 ***g*** and 4 °C. The supernatant was decanted into vials containing 10 ml of scintillation cocktail. Radioactivity was measured on liquid scintillation counter (Tri-Carb 2700TR, Packard Instrumental Co., Meriden, CT, USA). Non-specific binding was determined by the addition of a 100-fold molar excess of non-labeled E_2. Data were plotted as percent of [^{3}H]E_2 bound vs molar concentration of test compounds.

2.5. Androgen receptor binding assay

The protocol for the AR binding assay was based on that described by EDSTAC (US EPA, 1998). Male 8-week-old SD rats (260–300 g) were castrated. After 24 h, the rats were sacrificed. The ventral prostates were excised and homogenized with a Polytron homogenizer in ice-cold TEDG buffer A [10 mmol/l Tris–HCl, 1.5 mmol/l EDTA, 1 mmol/l DTT, 10 mmol/l $NaMoO_4$ (pH 7.4)] at 2.5 ml/g tissue. The homogenate was centrifuged for 60 min at 105,000 ***g*** and 4 °C. The supernatant is low-salt unoccupied cytosolic receptors (AR-rich cytosol).

25 μl of AR-rich cytosol was incubated with 3 nmol/l [^{3}H]R1881 and 100 μl of TEDG A buffer containing test compounds or DHT in glass tube at 4 °C for 18 h. Test compounds were dissolved in DMSO (final concentration 0.2%) and added to TEDG A buffer. After the incubation period, 0.5 ml of HAP slurry (made in 50 mmol/l Tris–HCl, pH 7.4) was added to each tube to separate the bound [^{3}H]R1881 from the free ligand. These tubes were incubated in an ice-cold water-bath for 20 min with mixing at 5-min intervals. Tubes were centrifuged for 3 min at 600 ***g*** and 4 °C. The HAP precipitation was washed three times with 2 ml Tris buffer (50 mmol/l, pH 7.4). After washing, ice-cold ethanol was added to each tube to extract the radiolabeled R1881, and tubes were mixed at 5-min intervals. Tubes were centrifuged for 10 min at 600 ***g*** and 4 °C. The supernatant was decanted into vials containing 10 ml of scintillation cocktail. Radioactivity was measured on liquid scintillation counter (Tri-Carb 2700TR, Packard Instrumental Co.). Non-specific binding was determined by the addition of a 100-fold molar excess of non-labeled R1881. Data were plotted as percent of [^{3}H]R1881 bound vs molar concentration of test compounds.

2.6. Uterotrophic assay

The protocol for the uterotrophic assay was based on that described by EDSTAC (US EPA, 1998), Odum et al. (1997) and Laws et al. (2000). Prepubertal 21-day-old female rats were dosed once per day for 3 days by sc injection with vehicle (corn oil), E_2 (0.04 mg/kg), *p*-nonylphenol (0.02, 0.2, 2, 20, 200 mg/kg), SM (20, 200 mg/kg), SD (0.02, 0.2, 2, 20, 200 mg/kg) or ST (0.02, 0.2, 2, 20, 200 mg/kg). The dosing volume was 5 ml/kg body weight. Adult 6-week-old female rats were ovariectomized under ether anesthesia and allowed to recover for 3 weeks. During the recovery period, vaginal smears from these rats were monitored 5 days prior to treatment to confirm a persistent diestrus state. These rats were dosed once per day for 3 days by sc injection with vehicle (corn oil), E_2 (0.01 mg/kg), NP (0.02, 0.2, 2, 20, 200 mg/kg), SD (0.02, 0.2, 2, 20, 200 mg/kg) or ST (0.02, 0.2, 2, 20, 200 mg/kg). The dosing volume was 1 ml/kg body weight. All compounds were dissolved or homogeneously suspended in corn oil. Both prepubertal and adult ovariectomized rats were anethetized using

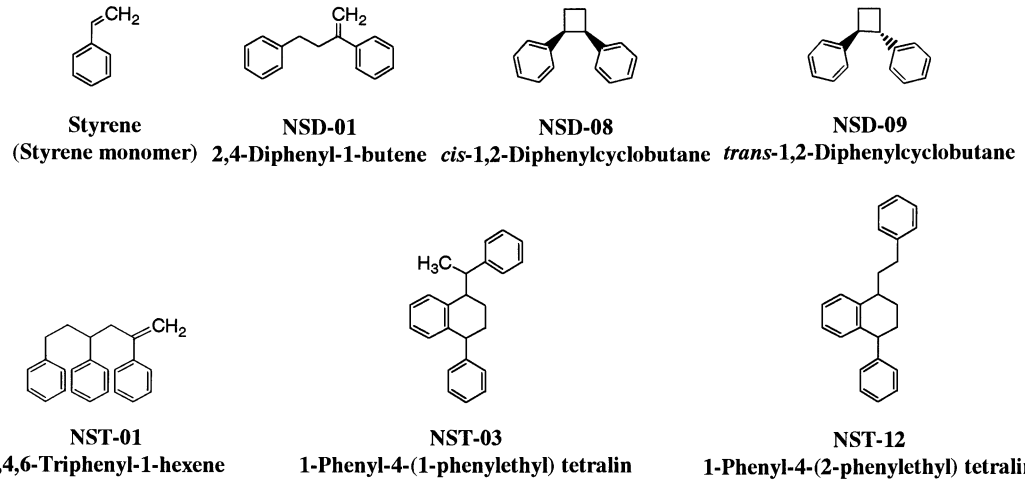

Fig. 1. Chemical structure of test compounds: styrene monomer (SM), styrene dimers (SD; NSD-10, 08 and -09) and styrene trimer (ST; NST-01, -03 and -12). NST-01 is recemic, NST-03 and -12 are optical inactive diastereomers. The purity of SM, SD and ST for assay are >98.5%.

ether and euthanized by exsanguination 24 h after final dose. Uteri were removed and adhering fat was trimmed away. The body of the uterus was cut just above its junction with the vagina and the bladder, and cut at the junction of the uterine horns with the ovaries. The uterus was then weighed.

2.7. Hershberger assay

The protocol for the Hershberger assay was based on that described by EDSTAC (US EPA, 1998). Immature 21-day-old male rats were sham-operated or castrated and allowed to recover for 7 days. After a recovery period, the castrated rats were dosed with TP (50 μg/body/0.2 ml) by sc injection, and with vehicle, flutamide (100 mg/kg), *p*,*p*′-DDE (200 mg/kg), SM (20, 200 mg/kg), SD (20, 200 mg/kg) or ST (20, 200 mg/kg) by oral gavage for 7 days. All compounds were dissolved or homogeneously suspended in corn oil. The dosing volume for oral gavage was 2.5 ml/kg body weight. All rats were anethetized using ether and euthanized by exsanguination 24 h after final dose, seminal vesicle, ventral prostate and levator ani plus bulbocavernosus muscle were removed and adhering fat was trimmed away, and weighed.

2.8. Thyroid hormone receptor binding assay

The protocol for the TR binding assay was based on that described by McKinney et al. (1987). A male 7-week-old SD rat (about 270 g) was sacrificed and the liver was excised. The liver was homogenized with a Polytron homogenizer in ice-cold buffer L1 [0.25 mol/l sucrose, 20 mmol/l Tris–HCl, 2 mmol/l $MgCl_2$, 0.1 mmol/l PMSF (pH 7.6)] and centrifuged at 1000 ***g*** and 4 °C for 15 min. The precipitation was suspended in a mixture solution (2.1 mol/l sucrose and 1 mmol/l $MgCl_2$) and centrifuged at 24,000 ***g*** and 4 °C for 90 min finally resulting in precipitation of a hepatic cell nucleus. The precipitation was suspended in the buffer L1 containing 0.5% Triton X-100 and centrifuged at 2500 ***g*** and 4 °C for 10 min. The precipitation was further suspended in buffer L2 [20 mmol/l Tris–HCl, 0.4 mol/l KCl, 2 mmol/l $MgCl_2$, 5% glycerol, 1 mmol/l EDTA, 1 mmol/l DTT (pH 7.6)] and centrifuged at 20,000 ***g*** and 4 °C for 20 min. The supernatant was assigned to the hepatic cell nucleus fraction TR.

A reaction mixture 0.5 ml of the hepatic cell nucleus fraction TR, 0.3 nmol/l [^{125}I]T_4 and the buffer L3 [20 mmol/l Tris–HCl, 50 mmol/l NaCl, 10% glycerol, 1 mmol/l EDTA, 1 mmol/l DTT (pH 7.6)] containing with test compounds or T_4 or T_3, was incubated at 4 °C for 18 h. The test compounds or T_4 or T_3 were dissolved in DMSO (final concentration 2%) and added to the reaction mixture. The concentration of the test compound, 10 μmol/l, is the concentration of the soluble upper limit in this buffer. After the incubation period, the mixture solution of 60% HAP and 0.2% Triton X-100 was added to each tube to separate the bound [^{125}I]T_4 from the free ligand. These tubes were incubated in an ice-cold water-bath for 20 min with mixing at 5-min intervals. Tubes were centrifuged for 3 min at 600 ***g*** and 4 °C. The HAP precipitation was washed three times with 2 ml Tris buffer (50 mmol/l, pH 7.4). After washing, ice-cold ethanol was added to each tube to extract the radiolabeled T_4, and tubes were mixed at 5-min intervals. Tubes were centrifuged for 10 min at 600 ***g*** and 4 °C. Radioactivity was measured on automatic gamma counter (CliniGamma 1272-001, LKB-WALLAC, Finland). Non-specific binding was determined by the addition of 1 μmol/l non-labeled T_4. The TR binding activity of the test compounds was evaluated from an antagonistic inhibitory activity of the test compounds against TR binding of [^{125}I]T_4.

2.9. Effects of styrene oligomers on serum prolactin level in ovariectomized F344 rats

The protocol for rat serum prolactin was based on that described by Steinmetz et al. (1997). 6-week-old F344 female rats (90–110 g) were ovariectomized and allowed to recover for 7 days. After recovery period, These rats were dosed with vehicle, E_2 (0.04 mg/kg), bisphenol A (20 mg/kg), *p*-nonylphenol (20 mg/kg), SM (20 mg/kg), SD (20 mg/kg) or ST (20 mg/kg) by sc injection for 3 days. These compounds were dissolved or homogeneously suspended in corn oil. The dosing volume was 1 ml/kg body weight. After 24 h of the final dose, rats were decapitated, trunk blood was collected, and the serum was analyzed for prolactin concentration using rat prolactin EIA system (Amersham International plc, Buckinghamshire, UK).

2.10. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). The statistical significance between multiple groups was analyzed by one-way analysis of variance (ANOVA). When the *F* test was significant, it was followed by Dunnett's multiple comparison test. In the case of the TR binding assay, the statistical significance was analyzed by Student's *t*-test or Aspin–Welch test. The significance level for all tests was set at $P < 0.05$.

3. Results

3.1. ER binding assay

E_2 and DES inhibited the binding of [^{3}H]E_2 to ER in a dose-dependent manner, exhibited great affinity for the ER, and IC_{50} values were 2.59 and 3.05 nmol/l, respectively (Fig. 2). *p*-Nonylphenol and bisphenol A,

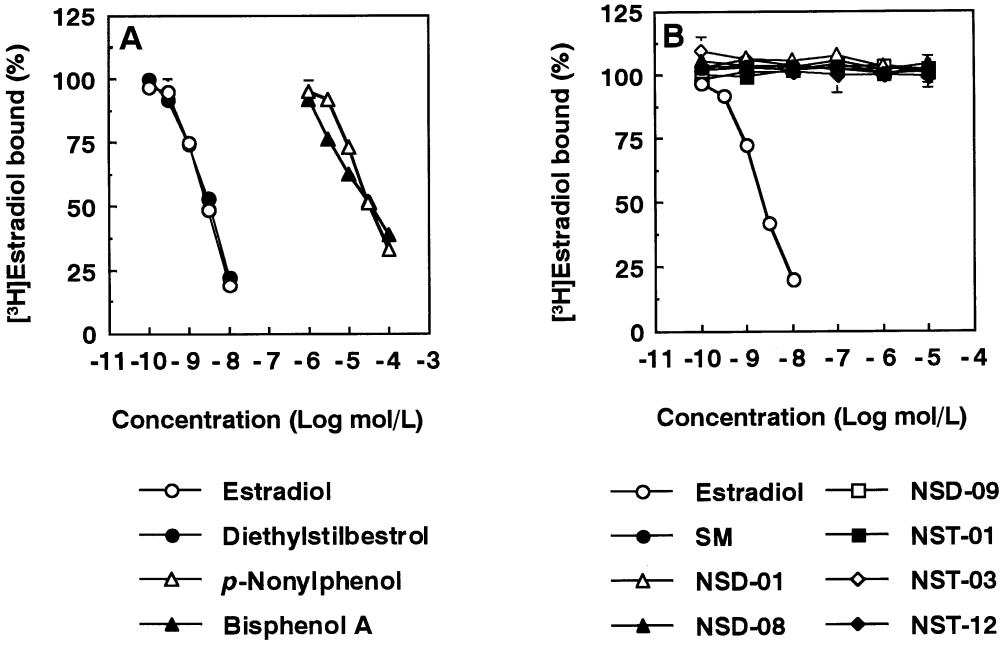

Fig. 2. Estrogen receptor binding assay of styrene monomer (SM), styrene dimers (NSD-01, -08 and -09) and styrene trimers (MST-01, -03 and -12). Rat uterine cytosol was incubated with 2 nmol/l [^{3}H]estradiol and test compounds. After incubation for 18 h at 4 °C, the receptors were separated and the radioactivity was counted. Each value represents the mean±S.D. of triplicate assays.

which have been reported to have estrogenic activity (Soto et al., 1991, 1995; Krishnam et al., 1993), inhibited [^{3}H]E_2 binding to ER in a dose-dependent manner, but they have a weaker affinity than E_2. The IC_{50} value of *p*-nonylphenol and bisphenol A were 37.2 and 33.7 μmol/l, respectively. In contrast, SM, SD and ST did not inhibit the binding of [^{3}H]E_2 to ER at the concentration ranging from 0.1 nmol/l to 10 μmol/l, and they did not bind to the ER.

3.2. AR binding assay

DHT inhibited the binding of [^{3}H]R1881 to AR in a dose-dependent manner, and the IC_{50} value was 4.21 nmol/l (Fig. 3). Flutamide and cyproterone acetate, which have been reported to be AR antagonists (Kelce et al., 1994), inhibited the binding of [^{3}H]R1881 to AR in a dose-dependent manner, but they have weaker affinity than DHT. The IC_{50} values of flutamide and cyproterone acetate were 66.4 and 0.272 μmol/l, respectively. In contrast, SM, SD and ST did not inhibit the binding of [^{3}H]R1881 to AR at the concentration ranging from 0.1 nmol/l to 10 μmol/l, and they did not bind to AR.

3.3. Uterotrophic assay

In prepubertal rats, E_2 (0.04 mg/kg) induced a significant increase in uterine weight as compared with control (Fig. 4). *p*-Nonylphenol (200 mg/kg) induced a significant increase in uterine weight (about 230% of control). In contrast, SM (20, 200 mg/kg), SD and ST (0.02, 0.2, 2, 20 and 200 mg/kg) did not induce any increase in uterine weight. Similar to prepubertal rats, in ovariectomized rats E_2 (0.001 mg/kg) prominently induced increase in uterine weight as compared with control (Fig. 5). Though *p*-nonylphenol (200 mg/kg) did not induce a significant increase, it has a tendency to increase (about 160% of control). In contrast, SD and ST (0.02, 0.2, 2, 20 and 200 mg/kg) did not induce any significant increase in uterine weight.

3.4. Hershberger assay

Seminal vesicle and levator ani plus the bulbocavernosus muscle weights in castrated rats following exposure to TP (50 μg/body) for 7 days, in comparison with the sham operated rats, significantly increased or showed an increasing tendency and the ventral prostate weight was equal to or reduced (Table 1). In TP-castrated rats, exposure via oral gavage for 7 days to *p,p′*-DDE and flutamide, reported as anti-androgenic chemicals (Kelce et al., 1995, 1997), seminal vesicle, ventral prostate and levator ani plus bulbocavernosus muscle weights were significantly reduced. In contrast, seminal vesicle weight in TP-castrated rats following exposure to NSD-01 (20 mg/kg) was significantly increased, while SM, SD and ST did not show any significant change in the weights of other organs.

3.5. TR binding assay

T_4 and T_3 inhibited [^{125}I]T_4 binding to TR in a dose-dependent manner, and their IC_{50} value was 7.00 and

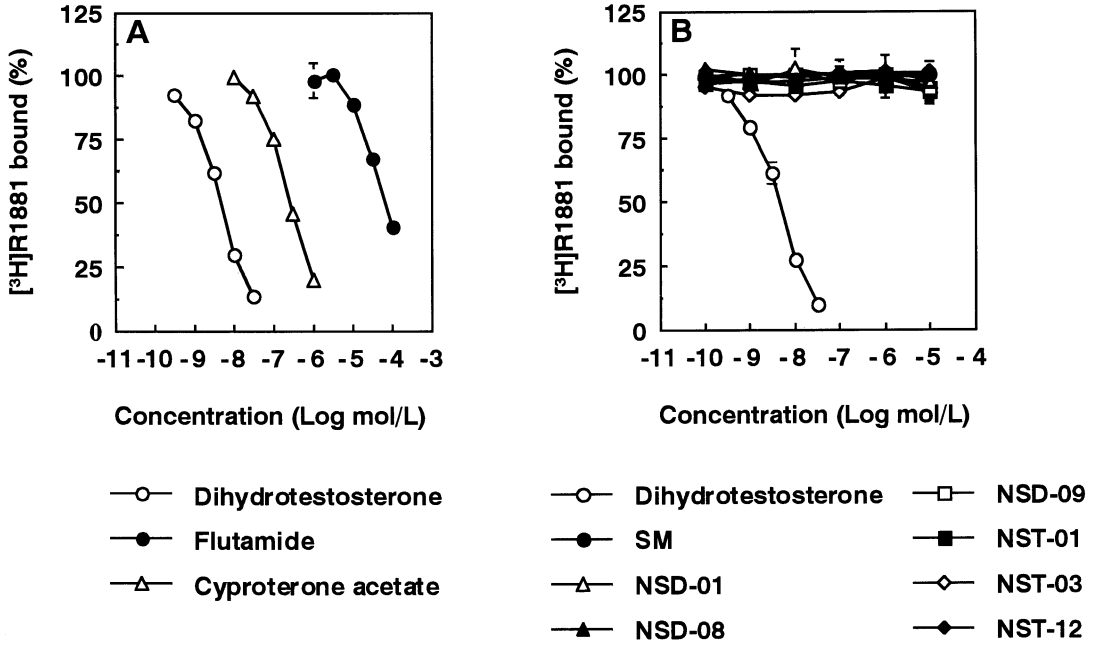

Fig. 3. Androgen receptor binding assay of styrene monomer (SM), styrene dimmers (NSD-01, -08 and -09) and styrene trimers (NST-01, -03 and -12). Rat prostatic cytosol was incubated with 3 nmol/l [^{3}H]R1881 and test compounds. After incubation for 18 h at 4 °C, the receptors were separated and the radioactivity was counted. Each value represents the mean ± S.D. of triplicate assays.

12.3 nmol/l, respectively (Table 2). In contrast, SM, SD and ST did not inhibit [^{125}I]T_4 binding to TR at a concentration of 10 μmol/l.

3.6. *Effects of styrene oligomers on serum prolactin level in ovariectomized F344 rats*

E_2 increased serum prolactin concentration about four times in ovariectomized F344 rats (Fig. 6). *p*-Nonylphenol and bisphenol A injection, with no significant change, increased serum prolactin concentration (about 124 and 144% of the control, respectively). In contrast, SM, SD and ST did not increase rat serum prolactin concentration.

4. Discussion

The safety of food-package containers is as important as the safety of the food it contains. In Japan the material used for plastic food containers is regulated by the Food Hygiene Law and specifications and standards are designated for PS. Similarly, PS is authorized by the United States Food and Drug Administration (FDA) as an indirect food additive for use in contact with food in Table 21 of the Code of Federal Regulations, Section 177.1640 (21 C.F.R. § 177.1640, "Polystyrene and rubber-modified polystyrene") and is commonly used in the manufacture of packaging materials including containers for many food products. However, it was recently reported that styrene oligomers remain in the PS container material used for instant noodles, and migrate from PS container into the food (Kawamura et al., 1998a,b,c,d). Additionally, styrene oligomers have been reported to have estrogenic activity (Prinsen, 1996).

Therefore, we investigated the endocrine-disrupting effects of SD and ST that eluted from PS containers and migrated into instant noodles, and that its structure has been determined (Yamada et al., 1999, 2000a,b). In the ER and AR binding assays, SM, SD and ST did not show any binding affinity for the ER and AR. Additionally, we have confirmed that these compounds do not show any estrogenic activity in the MCF-7 cell proliferation assay and the luciferase reporter gene assay (Ohno et al., 2001). These data suggest that SM, SD and ST cannot become agonist or antagonist to ER and AR. However, ER and AR distribute in the estrogenic target tissue (uterus, vagina, oviduct, mammary gland) and androgenic target tissues (prostate and seminal vesicle) as well as the hypothalamus and adenohypophysis at a high concentration to construct a feedback system of hormone biosynthesis. Therefore, it can be concluded that SM, SD and ST do not have influence on the sex hormone feedback system via these receptors.

A uterotrophic assay using prepubertal and ovariectomized adult rats was conducted to evaluate estrogenic activity in vivo. As a result, SM, SD and ST did not induce an increase in uterine weight, and also had no estrogenic activity in vivo. Laws et al. (2000) reported that the strength of hormone-like action differs according to the route of administration and the time from the final administration to uterus resection in the

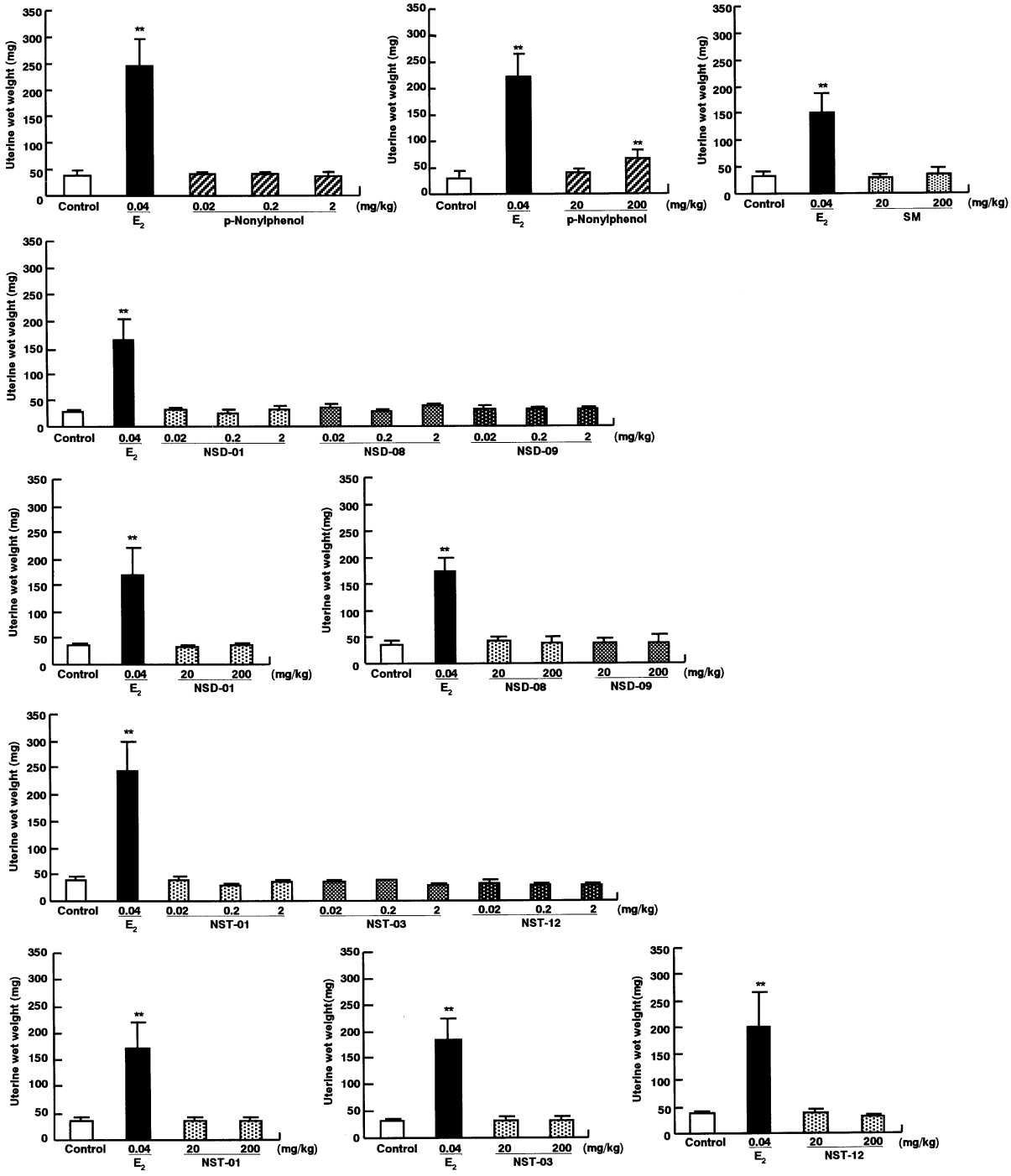

Fig. 4. Effects of styrene monomer (SM), styrene dimmers (NSD-01, -08 and -09) and styrene trimers (NST-01, -03 and -12) on uterine wet weight in prepubertal rats. 21-day-old rats were dosed by sc injection once a day for 3 days. Each value represents the mean±S.D. of five rats/treatment. **Significantly different from control ($P<0.01$).

uterotrophic assay, suggesting the importance of absorption and metabolism of a compound when exposed to animals, as well as the general toxicity test of a compound. With regard to this problem, although a detailed evaluation of styrene oligomers is considered necessary, from the results of the uterotrophic assay by oral gavage of Bachmann et al. (1998) and Fail et al. (1998), and the above-described results of the in vitro assay, it was suggested that oral gavage, similar to sc injection, may not show any estrogenic activity in vivo.

Next, anti-androgenic activity was evaluated by the Hershberger assay in the presence of testosterone treatment. Flutamide, which showed AR binding activity in vitro, reduced the prominently androgen-dependent seminal vesicle, ventral prostate and levator ani plus

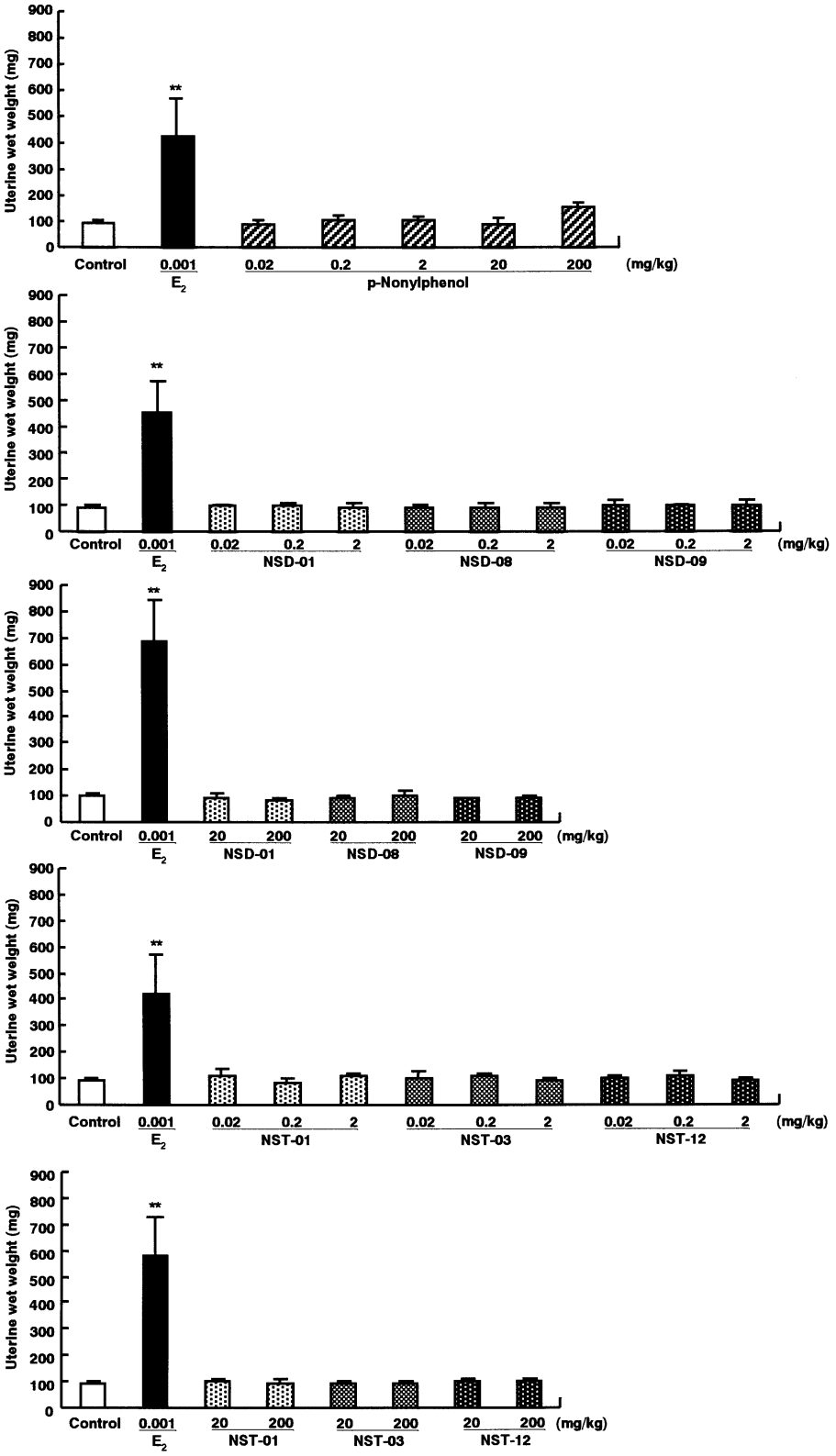

Fig. 5. Effects of styrene dimers (NSD-01, -08 and -09) and styrene trimers (NSD-01, -03 and -12) on uterine wet weight in ovariectomized adult rats. Ovariectomized adult rats were dosed by sc injection once a day for 3 days. Each value represents the mean±S.D. of five rats/treatment. **Significantly different from control ($P<0.01$).

bulbocavernosus muscle weights. Therefore, these results indicate that flutamide acts as an AR antagonist in vitro and in vivo. Under these conditions, SM, SD and ST, which showed no AR binding activity in vitro, did not reduce the weight of androgen-dependent organs. Therefore, this result suggests that SM, SD and ST do not act as an AR antagonist in vitro and in vivo.

As for the action of the endocrine-disrupting chemicals, an effect on hormone systems other than sex hormone, is feared, and in particular dioxins and PCB are considered to influence the thyroid hormone system via TR, and cause thyroid dysfunction (McKinney et al., 1987). It has been known that thyroid dysfunction leads to abnormal development, altered growth patterns, and a variety of physiological perturbations in animals. In the light of this background, we investigated TR binding activity and suggested that SM, SD and ST did not show any binding activity to TR. On the basis of these results, it can be presumed that SM, SD and ST do not influence growth and development of a body nor development of the central nervous system in the prepubertal period. In respect of growth and development, it has been reported that administration of styrene oligomers during gestation, including the major period of organogenesis and lactation, did not show any reproductive or developmental toxicity or impaired learning ability in their offspring (Nagao et al., 2000).

The effect on serum prolactin level was evaluated by using ovariectomized rats. Steinmetz et al. (1997) reported that E_2 increased serum prolactin level in ovariectomized F344 rats, and our data confirmed their results. No estrogenic activity of SM is evident from the above description in neither the in vitro nor the in vivo assay, and it was also reported by Soto et al. (1991, 1995). However, there is a report describing that serum prolactin level was increased by inhibition of dopamine synthesis in the brain by high exposure to SM in human (Mutti et al., 1984; Arfini et al., 1987). Therefore, it had

Table 1
Effects of styrene monomer (SM), styrene dimers (NSD-01,-08 and -09) and styrene trimers (NST-01,-03 and -12) on the weights of seminal vesicle, ventral prostate and levator ani plus bulbocavernousus in castrated, testosterone-treated immature male rats (Hershberger assay for anti-androgenic activity)

Exp. no.	Compound	Dose (mg/kg)	Castrate	TP	Organ weight (g)		
					Seminal vesicle	Ventral prostate	Levator ani muscle + bulbocavernosus muscle
1	Vehicle		–	–	43.7±8.7**	68.8±19.2	113.4±19.2**
			+	–	24.4±6.5**	9.5±2.7**	61.0±4.2 **
			+	+	91.6±15.4	69.1±15.6	183.9±13.6
	Flutamide	100	+	+	14.9±2.5**	11.7±1.2**	68.5±5.2**
	SM	20	+	+	97.3±12.4	70.3±6.9	178.2±17.6
		200	+	+	88.3±6.7	63.2±6.2	167.7±10.6
2	Vehicle		–	–	64.1±31.2	98.6±21.0*	147.4±30.2*
			+	–	18.7±4.8**	11.4±1.7**	80.2±8.2**
			+	+	86.4±9.3	69.8±7.8	197.2±17.8
	p,p′−DDE	200	+	+	38.8±7.4**	35.2±20.8**	128.8±34.7**
	NSD−01	20	+	+	102.7±18.0	77.3±12.5	218.6±22.5
		200	+	+	96.5±9.2	74.0±19.5	198.2±5.7
	NST−01	20	+	+	102.2±9.0*	58.0±15.7	216.1±42.7
		200	+	+	92.5±21.9	73.8±15.0	192.2±36.8
3	Vehicle		–	–	44.5±5.6**	65.8±12.3	130.6±12.1**
			+	–	16.3±1.1 **	9.6±1.7**	62.6±10.7**
			+	+	83.3±17.9	71.2±6.5	176.3±12.0
	p,p′−DDE	200	+	+	33.1±10.5**	29.7±9.1**	95.9±16.2**
	NSD−08	20	+	+	99.3±16.7	80.7±10.8	184.5±15.4
		200	+	+	90.1±19.5	77.5±10.7	175.2±18.2
	NSD−09	20	+	+	87.4±18.4	64.6±5.6	168.9±27.6
		200	+	+	90.2±26.5	75.8±7.5	178.8±23.1
	NST−03	20	+	+	90.3±24.0	71.8±7.8	181.4±11.4
		200	+	+	78.2±14.2	77.3±11.9	171.4±12.6
4	Vehicle		–	–	65.2±29.8	73.8±16.0	148.6±25.8
			+	–	15.2±2.1**	10.9±0.8**	70.5±8.7**
			+	+	93.4±15.3	62.6±15.5	174.0±28.0
	p,p′−DDE	200	+	+	39.1±6.2**	22.5±4.3**	115.9±20.1**
	NST−12	20	+	+	90.7±20.2	73.4±10.0	185.8±21.2
		200	+	+	92.4±22.2	63.6±9.8	177.2±11.9

Castrated immature male rats (28 days old) were treated with testosterone propionate (TP: 50 μg/body weight/day, sc) and each test compound (oral gavage) for 7 days. After 24 h of final treatment, the weights of seminal vesicle, ventral prostate and levator ani plus bulbocavernousus were measured. *, **: $P < 0.05, 0.01$ (vs castrated + TP). Each value represents the mean ± S.D. ($n = 5$).

been considered that there may be an endocrine-disrupting effect of SM, other than estrogenic activity. However, SM, SD and ST did not increase the rat serum prolactin level and therefore suggested that they had no effect on the system related to prolactin synthesis/release.

In conclusion, we investigated endocrine-disrupting effects of SD (NSD-01,-08 and-09) and ST (NST-01,-03 and-12) by ER, AR and TR binding assay, uterotrophic assay, Hershberger assay and rat serum PRL assay. As a result, it was clarified that SM, SD and ST did not show any binding affinity for ER, AR and TR, and also had no estrogenic or anti-androgen activity in vivo, and no effect on serum prolactin. Thus, it can be concluded that SM, SD and ST exhibit no apparent estrogenic, androgenic, anti-androgenic and thyroid activity.

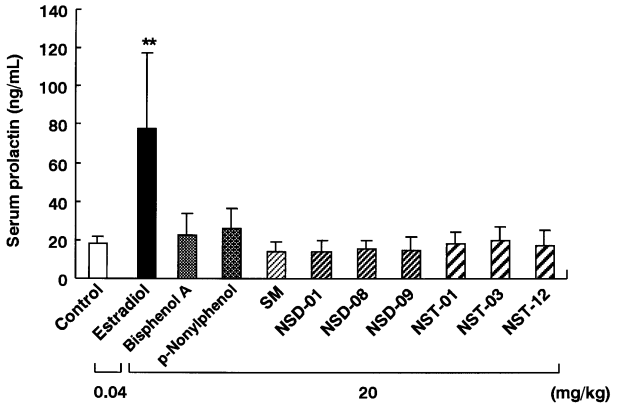

Fig. 6. Effects of styrene monomer (SM), styrene dimers (NSD-01, -08 and -09) and styrene trimers (NSD-01, -03 and -12) on prolactin release in ovariectomized F344 rats. Ovariectomized adult rats were dosed by sc injection once a day for 3 days. After 24 h of final injection, trunk blood was analyzed in duplicate for prolactin by EIA. Each value represents the mean±S.D. of five rats/treatment. **Significantly different from control ($P<0.01$).

Table 2
Thyroid hormone receptor binding assay of styrene monomer (SM), styrene dimers (NSD-01,-08 and -09) and styrene trimers (NST-01,-03 and -12)

Compound	Concentration (mol/l)	[^{125}I]Thyroxine bound (%)	IC_{50} (mol/l)
Thyroxine	10^{-9}	86.3±19.1	7.00×10^{-9}
	10^{-8}	36.3±0.7	
	10^{-7}	9.8±0.8	
Triiodothyronine	10^{-9}	89.1±6.4	1.23×10^{-8}
	10^{-8}	41.1±15.4	
	10^{-7}	22.8±3.1	
SM	10^{-5}	97.1±7.4	
NSD-01	10^{-5}	93.9±8.7	
NSD-08	10^{-5}	96.6±7.5	
NSD-09	10^{-5}	97.2±4.4	
NST-01	10^{-5}	106.5±6.3	
NST-03	10^{-5}	96.4±6.0	
NST-12	10^{-5}	96.0±5.3	

Nuclear thyroid hormone receptors obtained from rat liver were incubated with 0.3 nmol/l [^{125}I]thyroxine and test compounds. After incubation for 18 h at 4 °C, the receptors were separated and the radioactivity was counted. Each value represents the mean±S.D. of triplicate assays.

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Estimation of human exposure to styrene and ethylbenzene

Weici Tang, Ingrid Hemm, Gerhard Eisenbrand *

Department of Chemistry, Division of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, Erwin-Schroedinger-Str., D-67663 Kaiserslautern, Germany

Dedicated to Professor Dr K.J. Netter

Abstract

In the present studies, human exposure to styrene and to ethylbenzene (EB) is assessed on the basis of literature data. Total styrene and total EB exposure result from inhalation and from food intake. Styrene and EB inhaled represent the greatest proportion of the total intake. Styrene and EB content in food is mainly caused by migration from polymer packaging material. The daily styrene exposure is estimated to range from 18.2 to 55.2 μg/person, corresponding to an annual exposure of 6.7 to 20.2 mg/person. The daily EB exposure is estimated to be about 130 μg/person, corresponding to an annual exposure of 46 mg/person. Cigarette smoking is another important factor for styrene and EB intake by smokers.

Keywords: Styrene; Ethylbenzene; Human exposure

1. Introduction

Styrene is one of the most important industrial chemicals with a global production of more than 14 million t in 1992 (European Chemical Industry Council, 1994). Styrene is produced mainly by catalytic dehydrogenation of ethylbenzene and is used mainly for production of polystyrene and styrene copolymers. It is the second most widely used monomer for production of food-contact packaging polymers. Styrene is present in food and the environment. Human exposure to styrene occurs by routes depending on occupation, place of residence, and lifestyle.

In order to investigate potential health effects, several large epidemiological studies were performed to specifically address the potential carcinogenicity of styrene. In a historical cohort study conducted in several European countries, covering ca 40 000 workers from the reinforced polyester industry, where the styrene exposure was previously unusually high, no excess mortality was observed from all neoplasms. Mortality from neoplasms of the lymphatic and hematopoetic tissues increased with time of first exposure and average level of exposure to styrene but no consistent association with duration or cumulative exposure was observed in humans (Kogevinas et al., 1994). Similar results were also reported in some other cohort studies (Kolstad et al., 1994; Wong et al., 1994; Kolstad et al., 1995, 1996;

* Corresponding author. Fax: +49-631-205-3085.
E-mail address: eisenbra@rhrk.uni-kl.de (G. Eisenbrand)

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Gerin et al., 1998; Sathiakumar et al., 1998; Loughlin et al., 1999).

The DFG (Deutsche Forschungsgemeinschaft, 1999) in Germany has classified styrene into a newly defined category 5, comprising substances with carcinogenic potential considered to be so low that, provided that the maximum tolerable working place concentration is observed, no significant contribution to human cancer risk is to be expected.

Styrene has been found to be metabolized to styrene-7,8-oxide by cytochrome P450 2E1 (Guengerich et al., 1991). This key metabolite of styrene is able to bind covalently to DNA, causing formation of DNA-adducts (Phillips and Farmer, 1994; Schrader and Linscheid, 1997; Pauwels and Veulemans, 1998). Various in vitro and in vivo studies revealed styrene-7,8-oxide to be carcinogenic (Lutz et al., 1993; McConnell and Swenberg, 1993). On the bases of inadequate evidence for carcinogenicity in humans in conjunction with sufficient evidence for carcinogenicity in experimental animals, styrene-7,8-oxide was evaluated by the IARC as 'probably carcinogenic to humans' (group 2B) (International Agency for Research on Cancer, 1994).

In the present paper the potential human exposure to styrene for the general population will be estimated. Two main routes are known for human exposure to styrene: dietary intake with food and respiratory exposure to styrene from ambient air. The present estimation of styrene exposure levels is based on available data of styrene concentrations in food, as a constituent originally present in food or as a result of migration from packaging materials.

EB occurs in nature as a fraction of petroleum. It is also an important synthetic chemical that is produced in large quantities as a precursor for styrene and polystyrene. Since traces of residual EB might be present in polystyrene, it cannot be excluded that during food contact EB may migrate into food from polystyrene packaging material. The available information on human exposure to EB is collected and reviewed.

2. Styrene and EB concentration in food and human exposure

2.1. Styrene

2.1.1. Styrene concentration originally present in food

Styrene was first isolated from styrax, the resin of *Liquidambar orientalis*, arising from cinnamic acid by decarboxylation. High levels of styrene (up to about 40 ppm) were detected in cinnamon (Steele et al., 1994). The presence of styrene in certain plants and foods has been described. It is not clear however, whether styrene is formed endogenously or as a result of environmental contamination. Data reported about styrene concentrations in food are used therefore here without addressing the question of its origin.

Very low styrene concentration near the limit of detection (0.1 ppb) has been reported in many fruits and fruit products (Takeoka et al., 1986; Georgilopoulos and Gallois, 1987; Pfannhauser, 1988; Takeoka et al., 1988). Likewise, low styrene concentration near the detection limit was detected in various vegetables (Chung et al., 1983; Hayase et al., 1984). Styrene concentrations of about 5 ppb were observed for some beans (Fischer et al., 1979; Lovegren et al., 1979).

Styrene was found at the detection limit in cooked pork meat (Mottram et al., 1984), in cooked and roasted beef (MacLeod and Ames, 1986), in fried chicken (Tang et al., 1983) and guinea hen (Noleau and Toulemonde, 1988), as well as in fishes (Sabljic, 1987), in mussels (Yasuhara and Morita, 1987), and in eggs (Umano et al., 1990). Relatively high styrene concentration (100 ppb) was reported for Turkey sausage (Barbut et al., 1985). Low concentrations (0.1 ppb) were found in milk (Urbach, 1987) and cheese (Bosset and Gauch, 1993), whereas higher concentrations (up to 5 ppm) were detected in some moldy cheeses produced by some microorganisms (Adda et al., 1989).

Styrene was detected in honey (Graddon et al., 1979), in olives and olive oil (Biedermann et al., 1995), in coffee (Spadone et al., 1990) and cocoa (Gill et al., 1984). The styrene concentration in these foods was generally very low (0.1 ppb).

Exceptions were reported for olives. The styrene concentration in milled olive pulp increased significantly during 24 h storage at ambient temperature (from 27 to 230 ppb), but did not change significantly when stored in the refrigerator for a week, suggesting an endogenous origin of styrene in olives (Biedermann et al., 1995).

Styrene was detected in samples of drinking water in some early studies but this was not confirmed in several later studies (Newhook and Caldwell, 1993). According to the US Environmental Protection Agency (EPA), the styrene concentration in surface water was very low (0.1 ppb) and the drinking water was practically free of styrene (United States Environmental Protection Agency, 1987).

2.1.2. Styrene concentrations in food via migration from polymer packaging materials

In contrast to the low concentrations of styrene originally present in food, higher concentrations are observed as a result of styrene migration from polystyrene or styrene copolymer packaging materials. The migration of styrene monomer from polymer packaging materials into food varies with the physical and chemical characteristics of the polymer and of the food, the concentration of styrene monomer in the polymer, the diffusion coefficient, the diffusion distance, and the duration of the migration process (Murphy et al., 1992; Lickly et al., 1995a, 1997; Vandenburg and Gramshaw, 1997; Tawfik and Huyghebaert, 1998). The migration rate of styrene monomer into water or fatty food simulants was found to be directly proportional to the residual styrene concentration in polymers and to the storage time (Lickly et al., 1995a). The migrated styrene level in food increased with the fat content of food as a consequence of the good lipid solubility of styrene (O'Neill et al., 1994; Linssen and Reitsma, 1995; Tawfik and Huyghebaert, 1998).

The reported residual styrene concentrations in polystyrene or in styrene copolymers are generally within a range from <100 ppm to >3000 ppm. These residual concentrations are within the ranges given in official regulations (Bundesgesundheitsamt, 1989; US Food and Drug Administration, 1993). Concentrations of styrene reported for the majority of food packed in styrene polymers ranged from 5 ppb to 30 ppb, with up to 50 ppb in a few cases (Nerin et al., 1993; Varner et al., 1983; Ehret-Henry et al., 1994; Heikes et al., 1995; Kotiaho et al., 1995; Lau et al., 1995; Nerin et al., 1998).

2.1.3. Styrene concentrations in alcoholic beverages

The occurrence of styrene in wine has been studied in some detail as a consequence of styrene-associated off-flavour. Sponholz (1990) measured styrene concentrations in more than 100 German wine samples of the years 1983–1988 from Rheingau. The majority showed a styrene concentration of 1–3 μg/l with a maximal concentration of 8 μg/l. Hupf and Jahr (1990) examined white and red wines from Italy, Austria, former Yugoslavia, Romania, Hungary, Greece, USA, Portugal, Switzerland, Turkey, Tunisia and Germany. The majority (86%) of wines had styrene concentration up to 1 μg/l with a maximum of 8 μg/l. About one third of the samples were below the detection limit of 0.1 μg/l. In tanks of glass fiber reinforced styrene polymers, only small changes of styrene concentrations in wine during storage were observed. The migrated amount of styrene was negligible, compared to the styrene content originally occurring in such wines (Wagner et al., 1994).

The styrene concentration in beer was reported to be 10–200 ppb in a literature review by TNO, covering surveys on styrene contents in food published between 1965 and 1991 (Maarse, 1992). No specific source of contamination had been mentioned.

2.1.4. Estimation of human daily intake of styrene via food

Average per capita consumption figures of the general population in Germany are utilized for the estimation of human styrene intake via food (Deutsche Gesellschaft für Ernährung, 1996). According to the consumption profile, milk and milk products together add up to a total amount of 338 g/person/day. Fat and oil contribute another 72 g/person/day. If all these foods were packed in polystyrene materials, causing styrene contents re-

sulting from migration of 5–30 ppb, the daily styrene intake via such foods would reach 2–12 μg, which corresponds to an annual intake of about 0.7–4.4 mg/person.

Some additional styrene intake might originate from wine consumption. The annual consumption of wine is 24.5 l/person, according to the consumption profile for the German population. Supposing all wines had an average styrene content of 1–3 ppb, the total intake from wine reaches about 25–75 μg/year. Foods such as cereals, potatoes, fruits and beer are not taken into account because analytical evidence suggests their contribution to be minimal.

On the basis of this estimation, the average annual styrene intake via food consumption for the general population will be about 0.8–4.5 mg/person. Lickly et al. (1995b) calculated a styrene exposure of 9 μg in the daily food of the United States population which corresponds to an annual per capita intake of 3.3 mg styrene. A Canadian study calculated a daily intake of styrene from food ranging from <0.11 μg/kg up to <0.58 μg/kg, corresponding to an annual intake of <2.8 up to <14.8 mg/person (Newhook and Caldwell, 1993). In comparison, Lutz and Schlatter (1993) used an average human daily intake of 10 ng styrene per kilogram body weight for the Swiss population, corresponding to a daily intake of 0.7 μg/adult (70 kg body weight) and to an annual intake of 0.26 mg.

A somewhat more realistic estimation has to take into consideration that only a certain percentage of food is packed in polystyrene. Application of the US/FDA consumption factor (CF) that assumes that only ca 10% of the food is packed in polystyrene (CFpolystyrene = 0.1 (10%) results in an average annual intake of styrene of 0.08–0.45 mg/person or 1.1–6.5 μg/kg body weight for adults (70 kg body weight). This corresponds to an average daily intake of 0.2–1.2 μg/person or 3–17 ng/kg body weight.

2.2. Ethylbenzene

2.2.1. Ethylbenzene concentrations in foods

Only limited data are available for EB contents in non-packaged foods. EB was detected in fruits and fruit products, such as tomatoes and tomato products, apples, strawberries, and kiwi fruits (Dirinck et al., 1977; Chung et al., 1983; Hayase et al., 1984; Takeoka et al., 1986; Gorna-Binkul et al., 1996). The content of EB in positive samples was generally very low and near the limit of detection (0.1 ppb). EB is perceived not to be present as a natural plant constituent, but rather as a contaminant through air pollution (Dirinck et al., 1977).

EB was reported to be present in some vegetables (Gorna-Binkul et al., 1996) and vegetable seeds (Lovegren et al., 1979; Rembold et al., 1989) at concentrations near the detection limit. EB was also detected in some cereals. In a wheat grain sample with normal odor, 168 ppb and in a grain sample with musty odor 257 ppb of EB were reported (Wasowicz et al., 1988).

EB was detected in milk (Vallejo-Cordoba and Nakai, 1993; Imhof and Bosset, 1994) and dairy products, especially in cheese (Bosset and Gauch, 1993; Imhof and Bosset, 1994; Wood et al., 1994). The content of EB was below the detection limit in fresh high-quality milk, but was about 30 ppb in off-flavored poor-quality milk (Vallejo-Cordoba and Nakai, 1993). Trace amounts of EB were found in some samples of Swiss Emmental cheese (Imhof and Bosset, 1994). In low-fat dairy products like yoghurts and desserts, contents of EB below 4 ppb were reported (Ehret-Henry et al., 1994).

EB was reported to be present in meat (Snyder et al., 1996), fish (Murray and Lockhart, 1988; Vejaphan et al., 1988) and egg (Stein and Narang, 1990; Umano et al., 1990). Mean concentrations of EB in muscle of lake whitefish from several communities in Canada were between 11 and 24 ppb (Murray and Lockhart, 1988).

EB and other volatile aromatic hydrocarbons were found in virgin olive oil (Morales et al., 1994; Biedermann et al., 1995). In olives as delivered, the concentration of EB was 6 ppb, whereas in olives milled for 15, 30, 45, and 70 min, concentrations were 14, 20, 34, and 25 ppb, respectively. In the oil made from these olives, an EB concentration of 27 ppb was determined. It was suggested that olive oil contains EB and related aromatics, because olives might absorb aromatic

hydrocarbons from the air and store them to a substantial extent in the oil. A limit of 50 μg/l EB in extra virgin olive oil was proposed for Germany (Biedermann et al., 1995). EB was determined at concentrations of 4–20 ppb in rapeseeds, of 4–6 ppb in rapeseed oil, and of 4 ppb in walnuts (Biedermann et al., 1996). EB was also detected in other oils (Thompson, 1994; Takeoka et al., 1996) and margarine (Heikes et al., 1995). Drinking water has been found to be practically free of EB (Wallace et al., 1984).

2.2.2. Ethylbenzene concentration in foods via migration from polymer packaging materials

The presence of EB in foods appears primarily as a result of migration from polymer packaging materials, mostly from polystyrene. Commercial polystyrene resins may contain residual EB at quite low levels or at concentrations similar to styrene monomer, depending on the technical process used (Durst and Laperle, 1990). EB was found in 41 of 44 samples of polystyrene products with a median concentration of 50 ppm (range: 8–473 ppm) and in all 12 samples of styrene graft and copolymer products with a median concentration of 84 ppm (range: 61–202 ppm) (Hempel and Rüdt, 1988).

The concentrations of EB migrating from polystyrene cups into foods varied with the type of food stuffs they contained. In pork meat cooked in thermoset polyester dishes with a residual content of 25 ppm (1.5 h at 175°C in a microwave oven) <6–34 ppb of EB were found (Gramshaw and Vandenburg, 1995). Concentrations of EB migrating from thermoset polyester dishes (residual concentration: 8.3–29.3 ppm) into olive oil (175°C for 2 h) were 600–900 ppb (Jickells et al., 1990). The migration rate was found to primarily depend on the fat content of the food (Jickells et al., 1992). In eggs stored in polystyrene package material EB concentrations from 4 ppb up to 28 ppb were found (Matiella and Hsieh, 1991).

EB was identified in 234 table-ready foods with an average content of 14.6 ppb (range 6.4 up to 38.7 ppb) with the highest level found in margarine (Heikes et al., 1995). Off-flavors in the rind of Emmental cheese were found to be caused by volatile compounds, including EB, from an epoxy resin coated surface in the ripening cellar (Bosset and Gauch, 1993).

2.2.3. Estimation of human daily intake of ethylbenzene via food

The limited data available for EB contents in food make it difficult to estimate the human daily intake of EB via food. Apart from some rare cases of exceptionally high levels in food reported so far, the content of EB reported for the majority of food ranged from 5 ppb up to 20 ppb.

The total consumption of meat, fish, milk, dairy products, eggs, vegetables, butter, margarine and oils together amounts to about 1 kg/day/person. If one assumes the worst case that all food mentioned above is packaged in polystyrene and contains 5–20 ppb EB, as a result of migration an average daily EB intake of 5–20 μg/person or 0.1–0.3 μg/kg body weight is estimated for adults. Applying the US/FDA consumption factor, the average daily intake of EB is estimated to be 0.5–2 μg/person or 0.01–0.03 μg/kg body weight for adults. This corresponds to an average annual intake of 0.2–0.7 mg/person or 2.6–10 μg/kg body weight.

3. Styrene and ethylbenzene in environment and human exposure

3.1. Styrene

3.1.1. Styrene concentration in air

Styrene is widely spread in the atmosphere. Its presence in air is due principally to emissions from industrial production of styrene and styrene polymers and to combustion and incineration of styrene polymer-containing garbage. Further sources are emissons by coal-fired power stations, vehicle exhaust and cigarette smoke. Styrene in the environment represents a direct as well as an indirect exposure for humans. Styrene has been detected in several locations of different countries, including forests and mountains (Helmig et al., 1989), urban air (Guicherit and Schulting, 1985), highway tunnels (Dannecker et al., 1990), and sanitary landfills (LaRegina et al., 1986). Concen-

trations of styrene in outdoor air were often found to be below 1 μg/m^3, except for outdoor air near sanitary landfills or in industrial areas, especially in those with styrene production (LaRegina et al., 1986; Yang and Chen, 1987).

For human exposure, styrene concentration in indoor air is more important than that in outdoor air. Lifestyles have changed significantly in the last decades, especially with respect to the proportion of time people spend indoors. Most people spend about 60% of their day at home, 30% at work, 5% in transit and only 3% outdoors. Many of the elderly spend in excess of 95% of their time in rooms (Berry, 1989). Therefore, human exposure to styrene via contamination of indoor air needs consideration due to this time factor (Wallace and Clayton, 1987).

Pellizzari et al. (1986) have compared the concentrations of volatile organic compounds, including styrene, in indoor and outdoor air in different geographic areas in the USA. Results showed that the median concentrations of styrene in indoor air were 0.4–3.6 μg/m^3, with maximal levels of 3.7–54 μg/m^3, whereas those in outdoor air were only 0.3–4.2 μg/m^3, with maximal levels of 1–11 μg/m^3. Similar styrene concentrations were reported for the indoor air in Northern Italy (De Bortoli et al., 1986). In most studies, styrene concentrations in indoor air exceed those in outdoor air, indicating an important contribution of indoor sources to the exposure of the general population.

Personal air sampling can give a more precise assessment of human exposure to styrene, since personal samplers are carried by each participant during normal daily activities (Wallace, 1986). Median concentrations in personal air were found to be 1–3 μg/m^3. The concentrations of styrene in personal air were generally higher than in outdoor air.

Cigarette smoking considerably contributes to the styrene concentration in indoor air (Darrall et al., 1998). Styrene concentrations of about 3 mg/m^3 have been measured in cigarette smoke (Dmitriev et al., 1983), correlating well with the condensate levels. Cigarettes with high condensate levels gave 10 μg styrene in smoke per cigarette, whereas cigarettes with low condensate levels only gave 0.1–1 μg styrene in smoke per cigarette (Higgins et al., 1983; Byrd et al., 1990). Styrene concentration in indoor air of smokers was found to be higher than that of non-smokers, especially in fall and winter.

3.1.2. Estimation of the daily respiratory intake of styrene

Guicherit and Schulting (1985) estimated an average respiratory intake of styrene for the Dutch population of 6.5 mg/person and year, corresponding to a daily intake of 18 μg/person. This corresponds to an average concentration of styrene in the atmosphere of 0.6 μg/m^3, on the assumption of a daily respiratory intake of 30 m^3 for both outdoor and indoor air together. Fishbein (1992) estimated the respiratory daily intake of styrene on the assumption of a daily respiratory intake of 10 m^3 at the work place and 20 m^3 at home or in an urban atmosphere. Estimated styrene concentrations of 0.3 μg/m^3 were given for urban atmosphere, 20 μg/m^3 for polluted urban atmosphere, and 0.3–50 μg/m^3 for indoor air, corresponding to a styrene intake of 6 μg, 400 μg, and 6–1000 μg, respectively. An average styrene concentration in outdoor and in indoor air of 0.6 μg/m^3, as estimated by Guicherit and Schulting (1985) appears too low, compared with the median concentrations of styrene in person air (Wallace et al., 1988b) of 1–3 μg/m^3. We calculate here with an average styrene concentration in outdoor and in indoor air of 1–3 μg/m^3. The average concentrations of styrene in breath air were found to be about 40% of the inhaled air concentrations (Wallace et al., 1988a), which means that 40% of the inhaled styrene are exhaled. The daily respiratory intake of styrene therefore is calculated to be 18–54 μg/day, corresponding to 6.6–19.7 mg/year for the majority of the general population.

Styrene concentration in breath air was found to be significantly higher for smokers (1.1 μg/m^3) than for non-smokers (0.3 μg/m^3) (Hartwell et al., 1987; Brugnone et al., 1989).

3.2. Ethylbenzene

3.2.1. Ethylbenzene concentration in air

EB was found in 75–90% of air samples at measurable concentrations (Wallace et al., 1986).

Its presence in air is due mainly to industrial emissions. The concentration of EB in outdoor air is largely dependent upon the sites of sample collection. Concentrations of EB in outdoor air of different geographic areas in the USA ranged from <1 μg/m^3 up to 11 μg/m^3 (Pellizzari et al., 1986). Urban air concentrations were found to vary considerably with time, but median concentrations of EB overnight or at daytime were within the concentration range mentioned above throughout the seasons (Hartwell et al., 1987).

EB was also detected in indoor air (Bayer and Black, 1987; Berglund et al., 1989). In most studies, EB concentration in indoor air exceeded that in outdoor air, indicating substantial contribution of indoor sources to EB exposure (Wallace, 1986; Bozzelli et al., 1995). Median concentrations were in the range of about 2–10 μg/m^3, in the same order as those in outdoor air (Pellizzari et al., 1986; Minoia et al., 1996).

The concentrations of EB in person air were generally higher than in outdoor air (Wallace and Pellizzari, 1987). The most important factor to contribute to the exposure was smoking. Smokers displayed significantly elevated EB levels in breath (Wallace and Pellizzari, 1987; Wallace et al., 1989). The amount of EB delivered from one cigarette with 16 mg tar and nicotine was reported to be 8 μg (Wallace et al., 1987).

3.2.2. Estimation of the daily respiratory intake of ethylbenzene

Guicherit and Schulting (1985) calculated an annual respiratory intake of EB for Dutch people of 14.5 mg/person, based on a mean concentration of EB in ambient air of 0.20–0.65 ppb and a maximal concentration of 2.3–5.9 ppb. This corresponds to a daily intake of 40 μg in 20 m^3 air. The median amount of daily inhaled EB for primary school children in three Italian towns was calculated to be 15.5, 19 and 13 μg, based on indoor concentrations alone. Based on the indoor plus outdoor concentrations, 14.2 and 20 μg/person daily were calculated (Minoia et al., 1996).

Median concentration of EB in outdoor air was reported to be 5.2 μg/m^3 for USA in 1970–1980 (Environmental Sciences Research Laboratory, USA, 1982). This value agrees with the values reported in most studies. We use this value for both, outdoor and indoor air as a basis for estimation of the respiratory intake. On the assumption of a daily respiratory air intake of 30 m^3 for both, outdoor and indoor air together (Fishbein, 1992), the daily respiratory intake of EB is calculated to be 156 μg/person. The average concentrations of EB in breath air were found to be about 20% of the inhaled air concentrations (Wallace et al., 1988a), which means that 20% of the inhaled EB are exhaled. The daily respiratory intake of EB therefore is calculated to be 125 μg/person or 1.8 μg/kg body weight for adults. The resulting average annual intake corresponds to 45.6 mg/person or 652 μg/kg body weight.

4. Total human exposure to styrene and ethylbenzene

4.1. Styrene

The total human exposure to styrene is composed of the styrene intake via food and that via inhalation. Our estimated exposure levels are listed in Table 1.

The Canadian study estimated a daily total styrene intake for the Canadian general population ranging from <0.19 up to >0.85 μg/kg. Intakes from ambient air ranged from 0.004 up to 0.17 μg/kg and those from indoor air from 0.07 up to 0.10 μg/kg. Intake from food was calculated to range from <0.11 up to <0.58 μg/kg. The estimated intakes from drinking water and soil were negligible. Potential exposure from cigarette smoke, on the basis of the styrene content reported for mainstream smoke (10 μg/cigarette) and a smoking rate of 20 cigarettes per day, was estimated to be 2.86 μg/kg body weight for adults (Newhook and Caldwell, 1993). Our estimation for the general population agrees well with the total exposure data from the Canadian study. However, in our estimation inhaled styrene represents the majority of the exposure, whereas in the Canadian study styrene in food represents the major exposure source.

Table 1
Total human exposure to styrene and ethylbenzene for the general population

Intake		Styrene	Ethylbenzene
via Food	Daily μg/person (μg/kg)	0.2–1.2 (0.003–0.017)	0.5–2 (0.01–0.03)
	Annual mg/person (μg/kg)	0.08–0.45 (1.1–6.5)	0.2–0.7 (2.6–10)
via Inhalation	Daily μg/person (μg/kg)	18–54 (0.3–0.8)	125 (1.8)
	Annual mg/person (μg/kg)	6.6–19.7 (94.3–281)	45.6 (652)
Total	Daily μg/person (μg/kg)	18.2–55.2 (0.3–0.8)	ca 130 (ca 1.8)
	Annual mg/person (μg/kg)	6.7–20.2 (95.7–288)	ca 46 (ca 660)

The Styrene Information and Research Center (SIRC) estimated that most of the environmental exposure to styrene is from air. For risk assessment, the potential contamination of food and drinking water was ignored. In a worst case assessment, the daily human intake from regional exposure was estimated to be 0.67 μg/kg. This amount of inhaled styrene exposure is in agreement with the data in our estimation (Styrene Information and Research Center, 1997).

The respiratory styrene intake of smokers is higher than that of non-smokers. On the assumption that 5 μg styrene per cigarette will be inhaled additionally by smoking, the daily intake of styrene for smokers with 20 cigarettes per day accounts for 100 μg, corresponding to an annual intake of 36 mg. Fishbein (1992) gave a daily styrene intake of 400–960 μg with 20 cigarettes based on an estimated styrene concentration in cigarette smoke of 20–48 μg/cigarette. Cigarette smoking therefore represents an additional significant source of styrene intake for smokers.

4.2. Ethylbenzene

The total human exposure to EB is composed of the EB intake via food and inhalation and exceeds the styrene intake by a factor of about 2.36 (Table 1). Significantly higher exposure to EB results from smoking. The exposure to EB for smokers therefore is higher than for the general population, depending on the kind of cigarettes and on the numbers of cigarettes smoked.

5. Conclusions

On the base of our estimation, we conclude:

1. The styrene exposure for the general population is estimated to be in the range of 18.2–55.2 μg/person/day (0.3–0.8 μg/kg) or 6.7–20.2 mg/person/year (95.7–288 μg/kg), mainly resulting from inhalation and from food intake. The inhaled amount of styrene accounts for more than 90%, representing the majority of the total intake. The styrene content in food is mainly caused by migration from polymer packaging materials.
2. The daily EB exposure for the general population is ca 130 μg/person or ca 1.8 μg/kg/day, corresponding to an annual intake of ca 46 mg/person or ca 660 μg/kg. The majority with up to 99% of EB exposure is due to inhalation, only 1–2% of the total EB exposure being caused by food consumption.
3. Cigarette smoking is another important source of styrene and EB intake for smokers. The intake of styrene by smoking 20 cigarettes is more than the total daily intake from food and air. The additional exposure to EB caused by smoking is dependent on the kind of cigarettes and on the number of cigarettes smoked.

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