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Endocrine Disrupting Nonylphenols Are Ubiquitous in Food

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4-Nonylphenols (NPs) are common products of biodegradation of a widely used group of nonionic surfactants, the nonylphenol ethoxylates (NPEs). These compounds are known to be persistent, toxic, and estrogen active. There is a worldwide scientific and public discussion on the potential consequences of human long term dietary exposure to such endocrine disrupters. Despite numerous determinations of NPs in environmental samples no systematical reports exist relating to concentrations of NPs in food. We analyzed NPs in 60 different foodstuff commercially available in Germany. The results indicate that NPs are ubiquitous in food. The concentrations of NPs on a fresh weight basis varied between 0.1 and 19.4 μg/kg regardless of the fat content of the foodstuff. Based on data on German food consumption rates and these first analyses of NPs in food, the daily intake for an adult was calculated to be 7.5 μg/day NPs. For infants exclusively fed with breast milk or infant formulas daily intakes of 0.2 μg/day and 1.4 μg/day NPs, respectively, can be estimated.

Introduction

4-Alkylphenol ethoxylates (APEs) are a widely used class of nonionic surfactants with an annual worldwide production of about 650 000 tons with an estimated turnover of 600 million Euro. Nonylphenol ethoxylates (NPEs) are by far the prevalent subgroup of this surfactant class (*1*, *2*). They are synthesized by addition of ethylene oxide to NPs under alkaline conditions leading to NPEs with varying lengths of the ethoxylate chains (*3*). Technical NP consists of a complex mixture of isomers with differently branched structures of the nonyl side chain (*4*). While the use of NPEs in household detergents has diminished in some European countries in recent years they are still applied as dispersing agents in paper and pulp production, emulsifying agents in latex paints and pesticide formulations, flotation agents, industrial cleaners, cold cleaners for cars, and in the textile industry. Besides the predominant use of NPs for manufacturing NPEs they are also used in the form of tris(nonylphenol)phosphites as antioxidants in plastics.

Most of the NPEs are used in commercial and industrial cleaning products which are disposed of to sewer for subsequent treatment at sewage treatment plants. During the different steps of sewage treatment a complex biodegradation process of NPE takes place, leading to the formation of several biorefractory metabolites (*5, 6*). Especially the formed NPs are persistent and toxic, and after release in the aquatic evironment they accumulate in aquatic organisms (*7*−*9*).

In 1991 Soto et al. accidently discovered the estrogenic activity of NPs apart from their toxic properties (*10*). Investigations with different in vitro (vitellogenin gene expression (*11*), E-screen (*12*), yeast-screen (*13*)), and in vivo bioassays (rats (*14, 15*), rainbow trout (*16*), Japanese medaka (*17*)) have confirmed the estrogenic potency of NPs. NPs mimic the estrogenic effects of 17β-estradiol by binding to the estrogen receptor as they displaced [^{3}H]-17β-estradiol from its receptor in a competitive manner (*11*).

The presence of endocrine disrupting chemicals in the environment has caused increasing concern about their impact to wildlife and human health. They may be even related to increased hormone-dependent cancers and decreases in sperm quantity and quality in humans (*18*, *19*). One possible route of human exposure is residues of endocrine disrupting compounds in food (*20*).

Although much is known about the occurrence and fate of NPs in the different compartments of the environment (*2*) no systematic reports exist relating to concentrations of NPs in food. The aim of this study was to determine the endocrine disrupting NPs in foodstuff commercially available in Germany. The analytical method was designed to isolate and concentrate the NPs from a broad range of different food samples. The data obtained by this method should be used to estimate a daily intake of NPs.

Experimental Section

Chemicals. Technical NP and 4-n-NP was obtained from Fluka (85%, Buchs, Switzerland) and from Promochem (Wesel, Germany), respectively. 4-(2′-Nonyl)phenol (2′-NP) was obtained from K. H. Dötz/University of Bonn, Germany. A solution of 4-n-NP in methanol (1.25 μg/mL) served as internal standard. Stock solutions of NP at 219.8 μg/mL, 4-n-NP at 186.7 μg/mL, and 2′-NP at 410.0 μg/mL were prepared in cyclohexane and stored at 4 °C. *N*-Methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) was purchased from Chromatographie Service (Langerwehe, Germany). Anhydrous sodium sulfate (p.a.), sodium chloride (p.a.), nitric acid (p.a., 65%), and hydrochloric acid (Suprapur, 30%) were obtained from Merck (Darmstadt, Germany). Sodium sulfate was heated at 550 °C overnight and then stored in a desiccator. Solvents were either LiChrosolv (methanol and 2-propanol), Uvasol (acetonitrile, cyclohexane, isooctane, and hexane), or p.a. quality (acetone) available from Merck.

Food Samples. All foodstuffs were purchased from supermarkets in Germany and were packed as usual in trade (Table 1). They were stored unopened until analysis at 4 °C. Liquid or paste-like homogeneous food, e.g. beverages, milk, yogurt or baby food, were extracted without any pretreatment. Solid foodstuffs were homogenized by use of an Ultra-Turrax 18/10 (IKA-Werk, Staufen, Germany) or a blender 38BL41 (Waring, Connecticut) just before the extraction. The human milk was obtained from a breast-feeding 35 year-old woman.

Materials. The food samples were extracted applying steam distillation/solvent extraction using a steam distillation apparatus designed by Veith and Kiwus (*21*). This apparatus was fitted to a 2 L distilling flask at the bottom and to a reflux condenser at the top. Before every use the whole extraction unit was purified by heating of 400 mL of nitric acid under reflux for 2 h. After acid-free washing the purification was continued by successive heating of 300 mL of methanol and 300 mL of hexane under reflux for 2 h.

Extraction. The extraction solvent for the steam distillation/solvent extraction was obtained by dissolving 40 g of

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TABLE 1. Packaging Material of the Investigated Foodstuffs

glass	plastic bag/plastic foil	paper	plastic beaker (pp)[a]	aluminum foil/ aluminum tube	beverage carton	can
peanut creme	pasta	sugar	lard	mayonnaise	dairy products	tuna
gooseberry marmalade	liver sausage	butter	fresh cheese	chocolate	orange juice	pineapple
beer	apples	spinach	quark	chicken		
infant food	potatoes		creme fraiche	infant formulas		
	tomatoes			follow-on formulas		
	bread					
	coffee					

[a] PP: polypropylene.

sodium chloride and 2 mL of hydrochloric acid in 600 mL of water. The solvent was filled in the above-mentioned apparatus and purified by boiling and simultaneous extraction of the condensate with 20 mL of cyclohexane/isooctane (1:1, v/v) for 5 h. After replacement of the aqueous and overlying organic phase in the steam distillation apparatus with fresh solvents 15−80 g of food sample was placed in the distilling flask and suspended in the extraction solvent. Twenty microliters of 4-n-NP dissolved in methanol (1.25 μg/mL) was added as internal standard. The samples were then distillated for 5 h by simultaneous extraction with 20 mL of cyclohexane/isooctane (1:1, v/v). The organic extract was collected in a 100 mL volumetric flask together with the aqueous phase. The organic layer was separated and then dried with anhydrous sodium sulfate. Following drying the extract was reduced in volume nearly to dryness under a stream of nitrogen and redissolved in 500 μL of cyclohexane. The samples were stored at 4 °C in the dark prior to analysis. Procedural blanks were run before every food sample extraction.

Normal-Phase HPLC Cleanup. A supplementary cleanup step was performed on HPLC in order to remove coextracted organic compounds which interfered in the following GC-MS analysis. The HPLC system consisted of a pump (Merck-Hitachi, L-6200, Darmstadt, Germany), an interface (Merck-Hitachi, D-6000), a column thermostat (Merck-Hitachi, T-6300), a Rheodyne syringe-loading sample injector (Model 7125, Cotati, CA), and a fluorescence detector (Merck-Hitachi, F-1080). Normal-phase HPLC was performed with an aminopropylsilica column (Hypersil APS, 125 × 4 mm i.d., 3 μm particle size) which was purchased as prepacked column from CS-Chromatographie Service, Langerwehe, Germany. The eluents A and B were hexane and a mixture of hexane: 2-propanol (80:20, v/v), respectively. Elution was carried out with a linear program from 97% A and 3% B to 75% A and 25% B in 10 min at a flow rate of 1.0 mL/min. The composition of 75% A and 25% B was held for 4 min and then linearly programmed to the initial composition in 3 min. The detector was operated with an excitation wavelength of 260 nm and an emission wavelength of 300 nm. To determine the time window in which NP and the internal standard eluted in the HPLC (peak maximum ± 1 min) a standard solution of 4-n-NP in cyclohexane was injected before the sample solution. Then 150 μL aliquots of the sample solution were injected two times using a 100 μL sample loop, and each of the fractions containing the separated NPs and 4-n-NP were collected in 6 mL screw cap vials with tapered bottoms. The eluent was evaporated nearly to dryness under a stream of nitrogen and stored at 4 °C.

Derivatization. To the vials containing the evaporated HPLC fractions, 5 μL of the instrument standard 2′-NP (0.41 μg/mL in cyclohexane) and 300 μL of derivatization solution (MTBSTFA in acetonitrile, 1:100, v/v) were added. These solutions were then transferred to 500 μL crimp top vials and closed with aluminum crimp-caps with natural rubber/PTFE liners. The vials were held at 60 °C for 10 min.

Large-Volume-Injection GC/MS Analysis. The analyses of the derivatized samples were performed on a Finnigan MAT GCQ GC/MS/MS system (ThermoFinnigan, San Jose, CA). The GC was equipped with a deactivated fused-silica precolumn (J&W, 5 m × 0.25 mm, Folsom, CA) and a CP-Sil-8 CB capillary column (Varian/Chrompack, 30 m × 0.25 mm, 0.25 μm film thickness, Walnut Creek, CA). Helium was used as carrier gas with a column head pressure of 19 psi resulting in a gas flow velocity of 45 cm/s at 70 °C. Separation was performed using the following temperature program: 3 min at 70 °C, to 165 °C with 20 °C/min, to 250 °C with 4 °C/min, to 280 °C with 40 °C/min, and 5 min at 280 °C. The interface and source temperatures were set to 275 °C and 180 °C, respectively. Detection was carried out in electron impact ionization (EI) mode and full scan mode acquiring data from m/z 100 to 340. Rapid large-volume-injections of 100 μL of derivatized samples were performed with a CTC A200S autosampler (CTC Analytics) into an Optic 2-200 programmable temperature vaporization (PTV) injection system (ATAS Cambridge, Cambridge, U.K.) equipped with a wide-bore liner (3.5 mm i.d.) packed with silanized diatomite (ATAS Port 1). After injection the solvent was vaporized via the split exit of the injector at a solvent vent rate of 200 mL/min and a constant temperature of 70 °C with a total solvent vent time of 60 s. The injector was then programmed from 70 °C to 275 °C (33 min) at 5 °C/s with a splitless time of 2 min.

Calibrations, Response Factors, and Quantification. Calibration standards were prepared by silylation of mixtures containing 5 μL of a NPs solution (1.1 μg/mL, 4.4 μg/mL, and 10.99 μg/mL in cyclohexane), 5 μL of a 4-n-NP solution (0.37 μg/mL in cyclohexane), and 5 μL of a 2′-NP solution (0.41 μg/mL in cyclohexane) in the presence of 300 μL derivatization solution (MTBSTFA in acetonitrile, 1:100, v/v) as described above. These standards were used to generate response factors f_i in relation to the internal standard.

The unknown amounts of every single NP isomer i which were necessary to calculate the relative response factors f_i were determined by GC-FID of the above-mentioned standard solutions. A Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (FID) was used to this end. Sample injection was made in the splitless mode onto a 30 m × 0.25 mm i.d., 0.25 μm CP-Sil-8 CB capillary column. Injector and detector temperatures were 275 and 300 °C, respectively. The GC oven temperature program was identical to that of the GC-MS procedure. Column head pressure of the carrier gas (helium) was held constant at 19 psi.

Quantification of the NP isomers in the derivatized samples was carried out by the internal standard method using relative response factors f_i determined as described above. Each HPLC fraction was injected twice, and the average result of both fractions was used. For quantification of the NP isomers, integration of areas in reconstructed ion current (RIC) chromatograms was performed for those peaks that had the same retention times as the calibration standard as

well as concurrent mass spectra. Concentrations were calculated from the sum of these integrated areas in relation to the internal standard by use of relative response factors. From the total NPs amount of the sample the corresponding total NPs amount of the blank value (9 ± 3 ng NPs) was subtracted, and then the concentrations of NPs were calculated in relation to the fresh weight of the sample. The sample of every foodstuff was analyzed two times.

Recovery, Reproducibility, and Detection Limits. The recoveries of the internal standard 4-n-NP and the reproducibility of the complete analytical procedure were determined for all food samples and the appropriate procedural blanks by GC-MS in relation to the instrument standard 2′-NP. The results of duplicate extraction of every sample spiked with 25 ng 4-n-NP in methanol show efficient recoveries of 90−100% with relative standard deviations (RSD) of 2.7−4.6% for the procedural blanks but strong variations among the different food samples (12−126%, RSD = 3−25%). Especially for foodstuff with high fat contents such as butter, liver sausage, or mayonnaise low recoveries of < 25% were obtained. The calculated NP concentrations however were corrected on the basis of the internal standard recoveries.

The detection limit of the optimized method was evaluted from the procedural blanks spiked with 4-n-NP. The detection limit calculated as the sum of the mean blank values for NPs (9 ± 3 ng NPs) plus three times of the standard deviation was 18 ng NPs. As a prerequisite for the calculation of NP concentrations in foodstuff the corresponding absolute NP amounts had to be above the determination limit of 27 ng NPs (mean blank value plus six times of the standard deviation).

Results

Extraction and Clean-Up. Continuous steam distillation/solvent extraction already described in the literature for the enrichment of NPs from wastewater, sewage sludges, and soils (*22−24*) was also employable to the extraction of food samples. It provided good extraction yields and a remarkable selectivity due to the fact that only steam-distillable and cyclohexane-isooctane-soluble compounds were efficiently enriched. But it has to be emphasized that analysis of NPs in food demands high standards concerning the purity of the apparatus and chemicals. Therefore prior to analysis every apparatus was drastically cleaned by hot nitric acid to remove burnt food residues followed by thoroughly acid-free washing and determination of the blank values.

Despite the selectivity of the steam distillation/solvent extraction method the high complexity of the food samples resulted in extracts still containing a lot of matrix compounds which interfered with the different NPs in the GC-MS chromatograms. Therefore an additional cleanup step on normal-phase HPLC was introduced which proved to be an essential part of this method. The HPLC method utilized was a modified version of that used by Ahel and Giger (*22*). The elution of the analyte and the internal standard in one peak made an easy collection of the NP-containing fraction possible.

GC-MS with Rapid Large Volume Injection. The use of large volume injection via PTV injectors equipped with packed wide-bore liners effectively enhances the sensitivity of a GC system (*25*, *26*). In this study, a PTV injector with a wide-bore liner packed with silanized diatomite was used for large volume injection in GC-MS. A sample volume of 100 μL was rapidly injected at 70 °C at which the solvent evaporated and was discharged via the split exit, while the less volatile analytes were retained in the packed liner. After the solvent elimination step the trapped analytes were transferred to the column in splitless mode by rapid temperature-programmed heating of the injector. The duration of the solvent vent time and the temperature of the injector were rather critical and had to be thoroughly optimized. Closing the solvent vent too late resulted in extra loss of the analytes, closing it too early resulted in peak distortion due to excessive solvent vapor in the column.

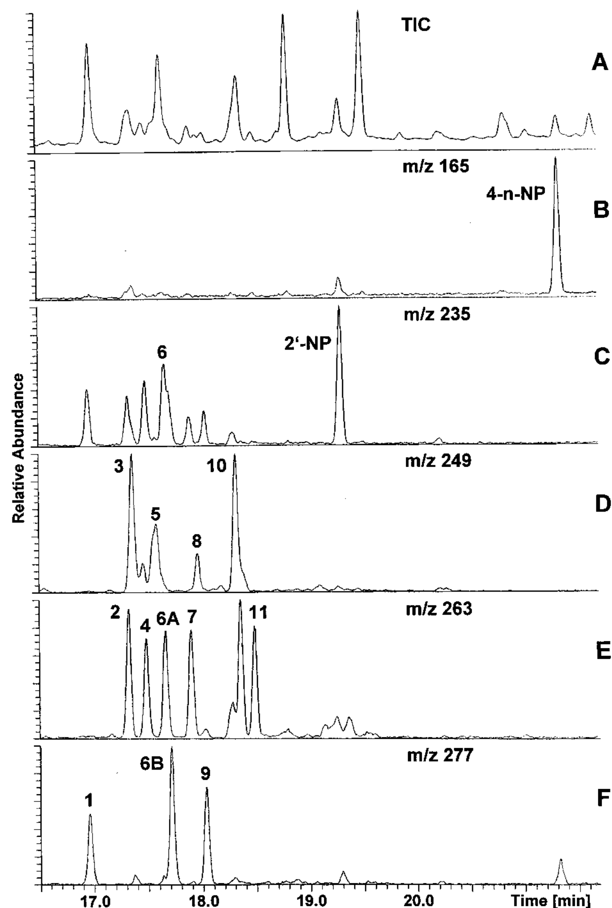

FIGURE 1. Total ion current (TIC) chromatogram (A) and reconstructed ion current (RIC) chromatograms (B−F) of silylated NPs derivatives from milk (10% fat) spiked with 4-n-NP and 2′-NP as standards.

Figure 1A shows the total ion current (TIC) chromatogram of an extract of condensed milk (10% fat). A typical peak pattern of technical NP, however, deriving from the separation of the different isomers of silylated NP by capillary GC even becomes obvious in the reconstructed ion current (RIC) chromatograms (*m/z* 235, 249, 263, and 277, Figures 1C−F). The peak at 19.16 min (Figure 1C) was derived from the instrument standard 2′-NP, while the peak at 21.17 min (Figure 1B) was attributed to the internal standard 4-n-NP. The peak patterns of NPs with respect to number and retention times of the peaks were identical in technical NP and the various foodstuffs. For calculation of the total NP concentrations in the food samples the 12 resolved peaks numbered in the different RIC chromatograms (Figure 1) were used.

The mass spectra of the silylated NP isomers are characterized by very weak molecular ions of *m/z* 334 and varying abundances of *m/z* 221, 235, 249, 263, 277, 291, and 305 due to the various branchings of the nonyl chains. In principle the fragmentation patterns of the silyl derivatives are very similar to those of the free phenols with respect to the homologous series of fragment ions being 14 mass units apart (*27, 7, 28, 4*). Primarily analysis of the mass spectra was difficult because of coelution of several NP isomers in individual peaks. Wheeler et al., however, achieved a better resolution of the NP isomers using a 100 m GC column (*4*). Comparison to their mass spectra indicated the presence of

TABLE 2. Elucidation of the Chemical Structures of the NP Isomers by Their EI Mass Spectra

Group	NP isomers *i*	Chemical structure[a]	Base peak *m/z*	Fragment peaks *m/z*
1	1, 6B	$tBu(Me)_2SiO$–(phenyl)– C_3H_7, C_4H_9	277	291 $[M - C_3H_7]^+$ 235 $[M - C_3H_7 - C_4H_9]^+$
2	2, 4, 6A	$tBu(Me)_2SiO$–(phenyl)– C_5H_{11}	263	305 $[M - C_2H_5]^+$ 249 $[M - C_5H_{11} - CH_3]^+$ 235 $[M - C_5H_{11} - C_2H_5]^+$
3	3, 5, 8, 10	$tBu(Me)_2SiO$–(phenyl)– C_6H_{13}	249	-
4	7, 11	$tBu(Me)_2SiO$–(phenyl)– C_5H_{11}	263	291 $[M - C_3H_7]^+$ 235 $[M - C_7H_{15}]^+$
5	9	$tBu(Me)_2SiO$–(phenyl)– C_4H_9	277	235 $[M - C_7H_{15}]^+$

[a] The wavy lines indicate the bonds which cleavage results in the formation of the base peak fragment ions.

five distinct groups of isomers (Table 2). Briefly, the spectra of group 1 isomers had a base peak at m/z 277 ($[M - C_4H_9]^+$) and two major peaks at m/z 291 ($[M - C_3H_7]^+$) and 235 ($[M - C_3H_7 - C_4H_9]^+$) indicating that an α-methyl-α-propyl configuration was most likely. The group 2 isomers exhibited mass spectra with a base peak at m/z 263 ($[M - C_5H_{11}]^+$), suggesting that they have an α-methyl-α-ethyl configuration. The presence of an ethyl group on the α-C-atom was supported by the occurrence of m/z 305 corresponding to $[M - C_2H_5]^+$. The third group of isomers showed mass spectra with a base peak at m/z 249 ($[M - C_6H_{13}]^+$) and no other considerable fragment ions indicating an α,α-dimethyl structure. The two isomers of group 4 produced a base peak at m/z 263 and a major peak at m/z 235 most likely for an α-methyl-β-methyl structure. The mass spectrum of the group 5 isomer had a base peak at m/z 277 and a major peak at m/z 235, suggesting that it has an α-methyl structure with a secondary β-C-atom. The mass spectrum of silylated 4-n-NP showed a base peak at m/z 165 ($[M - C_8H_{17} - C_4H_8]^+$), whereas silylated 2'-NP had a base peak at m/z 235 according to an α-methyl structure. The proposed structures of group 1, 2, and 3 isomers (Table 2) could be verified by work being undertaken on the structure elucidation of the technical NP isomers.

Application of GC/MS to the analysis of NPs in very complex matrices such as food offers high selectivity and sensitivity which could not be achieved with GC-FID and even HPLC. Moreover, the mass spectra of NP isomers not only confirm their presence but also allow proposals for their chemical structures.

Food Samples. Thirty-nine different food samples of a typical market basket of German people and 21 samples of different baby and infant food including breast milk were collected. For a representative selection of the food samples a national food consumption survey (*29*) as well as a study on German eating habits (*30*) were used. The data of these reports based on a survey on the eating habits of almost 25 000 German people from the Western part of the Federal Republic of Germany carried out from 1985 to 1988. Children under the age of four were disregarded. The different food products were divided into 24 groups, and the average daily consumption of products of each group were calculated (Figure 2). At least one food sample was selected from every group, and attention was paid only to buy very popular products.

All investigated food samples contained NPs in the concentration range of 0.1−19.4 μg/kg on fresh weight basis (Figure 3). Despite the strong lipophilic properties of NPs expressed by the logarithm of the octanol/water partition coefficient of 4.2 (31) high concentrations of NPs were not

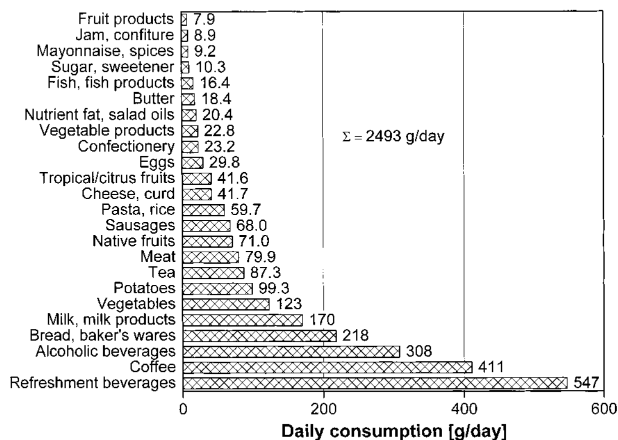

FIGURE 2. Average composition of food daily consumed by German adults. A total amount of 2493 g/day results from these data (*29*).

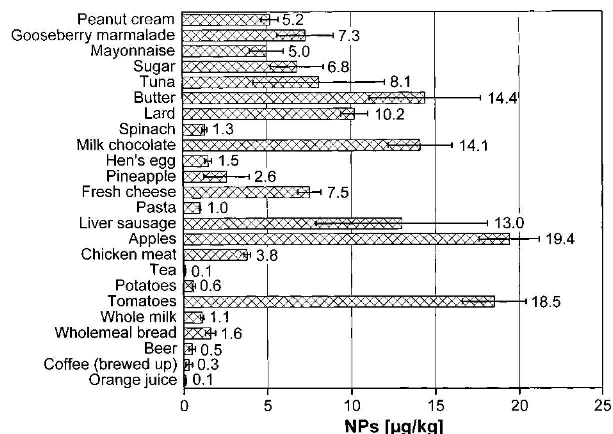

FIGURE 3. Concentrations of nonylphenols (NPs) in food samples from Germany selected on the basis of the 24 different food types in Figure 2. Concentrations (sum of all NP isomers, mean values with standard deviations, $n = 2$) are expressed on a fresh weight basis.

only found in fatty food like e.g. butter (14.4 μg/kg), lard (10.2 μg/kg), or liver sausage (13.0 μg/kg) but also in nonfatty food like e.g. marmalade (7.3 μg/kg), apples (19.4 μg/kg), or tomatoes (18.5 μg/kg). Moreover, several chocolate samples and milk products with varying fat contents were investigated. Results given in Figure 4 showed that the concentrations of NPs did not correlate with the fat contents. The four milk products marked in Figure 4 which considerably diverged from each other in their fat contents (0.3−30%) had almost the same concentrations of NPs (0.4−0.7 μg/kg).

With respect to risk assessment of dietary components infants must be considered as separate groups distinct from adults. First, the types of foods and levels of intake are considerably different and second, very young organisms may show qualitatively and quantitatively distinct sensitivities regarding to endocrine disrupting chemicals. Therefore, several infant food products including formulas on the basis of powdered milk, breast milk, and baby food without meat and with meat were also analyzed for NPs (Figure 5). NPs were present in all of these investigated samples varying in the range of 0.2−4.0 μg/kg. Concentrations of NPs in formulas (referring to the prepared formula in water) were higher than in breast milk.

Discussion

Our survey indicates that NPs are ubiquitous in food products commercially available in Germany (Figures 3−5). The widely varying concentrations of NPs independent of the fat contents

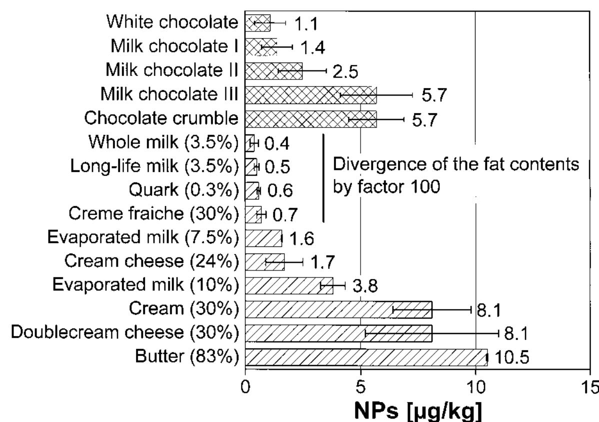

FIGURE 4. Concentrations of nonylphenols (NPs) in chocolate samples and different milk products commercially available in Germany. The values in brackets are the fat contents according to the manufacturers information. Concentrations (sum of all NP isomers, mean values with standard deviations, $n = 2$) are expressed on a fresh weight basis.

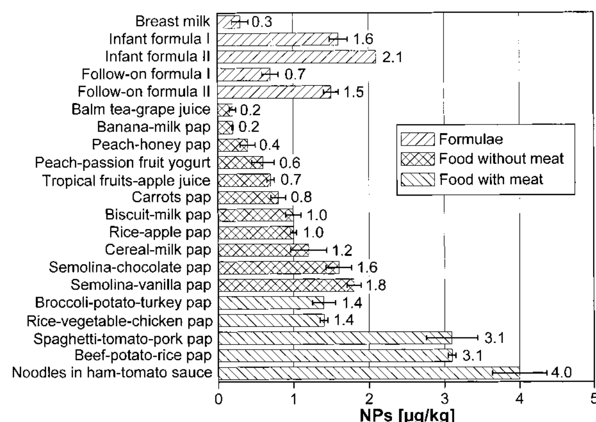

FIGURE 5. Concentrations of nonylphenols (NPs) in breast milk and infant food commercially available in Germany. Concentrations (sum of all NP isomers, mean values with standard deviations, $n = 2$) are expressed on a fresh weight basis.

and the packaging materials of the food samples imply that NPs get into food on miscellaneous pathways and at different stages of food production. A part of it could originate from NPEs which are used as nonionic surfactants in disinfectants and cleaning agents or as emulsifiers in pesticide formulations. After application in stables or food industries and in agriculture, respectively, degradation of NPEs could lead to the accumulation of NPs in food. In particular, the high concentrations of NPs in apples and tomatoes could be consequently attributed to pesticide application. Then, the lipophilic NPs would be accumulated in the wax coats of the fruits and vegetables, respectively. Another source might be plastic packaging materials from which NPs, used for example in tris(nonylphenol)phosphite as antioxidant, could migrate into food (*32*).

The average daily intake of NPs via food for a German adult calculated from the data in Figures 2–4 was 7.5 μg/day. For infants exclusively feeded with breast milk or infant formulas (daily consumption of 750 mL of breast milk or infant formula) average daily intakes of NPs of about 0.2 μg/day and about 1.4 μg/day, respectively, can be estimated (Figure 5). An average estimation of the daily intake of NPs for older infants will be possible after receipt of a food consumption survey and further investigations of infant food.

Although all food samples were only purchased from German supermarkets, many of them originated from abroad and others are worldwide consumed; therefore, the calculated daily intakes are also important to people from many other countries.

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