Regulatory Toxicology and Pharmacology Volume 44, Issue 2 , March 2006, Pages 161-171

doi:10.1016/j.yrtph.2005.10.002

Consumer product in vitro digestion model: Bioaccessibility of contaminants and its application in risk assessment

Esther F.A. Brandon*, Agnes G. Oomen, Cathy J.M. Rompelberg, Carolien H.M. Versantvoort, Jacqueline G.M. van Engelen and Adrienne J.A.M. Sips

Centre for Substances and Integrated Risk Assessment, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands

* Corresponding author. Fax: +31 0 30 2744475.

Abstract

This paper describes the applicability of in vitro digestion models as a tool for consumer products in (ad hoc) risk assessment. In current risk assessment, oral bioavailability from a specific product is considered to be equal to bioavailability found in toxicity studies in which contaminants are usually ingested via liquids or food matrices. To become bioavailable, contaminants must first be released from the product during the digestion process (i.e. become bioaccessible). Contaminants in consumer products may be less bioaccessible than contaminants in liquid or food. Therefore, the actual risk after oral exposure could be overestimated. This paper describes the applicability of a simple, reliable, fast and relatively inexpensive in vitro method for determining the bioaccessibility of a contaminant from a consumer product. Different models, representing sucking and/or swallowing were developed. The experimental design of each model can be adjusted to the appropriate exposure scenarios as determined by the risk assessor. Several contaminated consumer products were tested in the various models. Although relevant in vivo data are scare, we succeeded to preliminary validate the model for one case. This case showed good correlation and never underestimated the bioavailability. However, validation check needs to be continued.

1. Introduction

Contaminants in consumer products may pose a threat to human health. Especially the behaviour of young children to put things into their mouths, might pose them at a higher exposure compared to adults. However, the total amount of a contaminant in a consumer product does not always reflect the amount that is available to the body. Only a fraction of the contaminant in a consumer product may be bioavailable after oral exposure and exert its toxic action. For that reason, there is a demand for simple tools that enable the estimation of internal exposure of contaminants caused by mouthing toys or other consumer products specifically by children. Furthermore, current risk assessment is in most cases based on the bioavailability of a contaminant from food or liquid and does not incorporate the difference in bioavailability of a contaminant in a consumer product versus food or liquid. Better insight into oral exposure to a contaminant from a consumer product will lead to a more accurate health risk assessment for that specific situation.

Oral bioavailability (Fig. 1) is defined as the fraction of an ingested contaminant in a certain matrix that reaches the systemic circulation. We distinguish three processes in oral bioavailability: (1) release of the contaminant from its matrix during digestion in the gastrointestinal tract (bioaccessibility, F_B), (2) absorption of the bioaccessible fraction (F_A), and (3) metabolism in the intestine and liver (first pass effect, F_M). Release of the contaminant from the consumer product is required for transport across the intestinal epithelium and bioavailability of a contaminant to the body. The matrix of ingestion mainly affects the bioaccessibility. Absorption and metabolism depend more on the compound specific properties and physiology and, therefore, the matrix is expected to have less influence on these processes. However, in some cases, the ingested matrix has been shown to affect the transport of the contaminant across the intestinal epithelium (Wienk et al., 1999). For example, studies have indicated a decreased absorption of lead in the presence of calcium ions from the matrix. We therefore consider the bioaccessibility (F_B) of a contaminant as an indicator for the maximal oral bioavailability of the contaminant, which can be used for realistic worst case risk assessment of a contaminant in a consumer product.

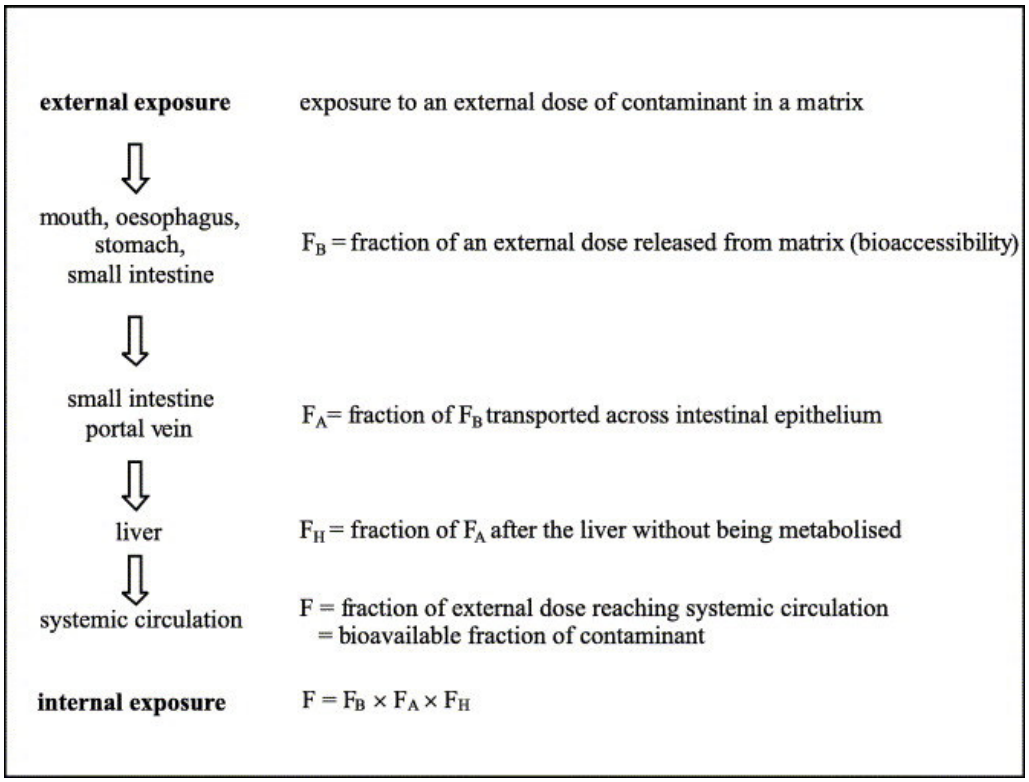

Fig. 1. Processes in oral bioavailability.

Toys form an important group of consumer products for which it is relevant to gain insight into oral bioavailability following mouthing or swallowing. Regulations for chemicals in toys are formulated in order to protect children's health and prohibit the presence of contaminants above a certain level or consider a migration limit of the contaminant (NEN-EN 71-3:1995/C1:2002 en, 2002, NEN-EN 71-7:2002 (en), 2002, NEN-EN 71-9:2003 Ontw. en, 2003, Commission Directive 1999/815/EC, 1999, Commission Directive 93/11/EEC, 1993 and Commission Directive 88/378/EEC, 1988). The European Commission recently banned (June 2005) the presence of six phthalates [di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBP), di-iso-nonyl

phthalate (DINP), di-iso-decyl phthalate (DIDP), and di-*n*-octyl phthalate (DNOP)] in concentrations of more then 0.1% in toys for children of all age categories and not only for children under the age of three due to endocrine disruption effects (Commission Directive COM 2005 (143), 2005). Recently, the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) gave an opinion on "Assessment of the bioavailability of certain elements from toys" (CSTEE, 2004). In this opinion, the CSTEE recommends to change the definition for bioavailability from 'the soluble extract having toxicological significance' into 'the amount of each element in the toy which could be absorbed into the systemic circulation of a child'. This new definition is in line with the definition of bioaccessibility. The test methods described in the above mentioned EU legislation are mainly based on non-physiological conditions. To our opinion, this may lead to unrealistic values for bioavailability. Moreover, these test methods represent mouthing and swallowing separately. In a physiological based test these two processes can be tested, if relevant, in the same system in a chronological way.

The aim of our study was to develop an in vitro digestion model for consumer products to simulate the different oral exposure scenarios for children, based on human physiology (chemical composition of digestive fluids, pH and residence time periods typical for each compartment). Such a model can be used in risk assessment for a more refined exposure scenario. Within our group, in vitro digestion models were already developed to assess the bioaccessibility of contaminants from soil and food in the gastrointestinal tract (Oomen et al., 2003a and Versantvoort et al., 2005). These models were used as starting point for the development of an in vitro digestion model for consumer products for sucking and/or swallowing. The new models were developed in close collaboration with risk assessors. Different contaminants from different consumer products (spiked and real-life case material) were tested for their bioaccessibility in the in vitro digestion models, e.g., azo dyes from textile and lead from paint scraped of tops.

2. Material and methods

2.1. Materials

Glucuronic acid was obtained from Fluka (Buchs, Switzerland) and mucin from Roth (Karlsruhe, Germany). Lipase and bile were provided by Sigma (St. Louis, MO, USA). All other chemicals for the digestive fluids were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2. In vitro digestion models

The general set-up of the digestion models will be described in the next four sections and is schematically shown in Fig. 2. The composition and preparation of non-stimulated (under fasted conditions and no stimuli for secretion like sucking) and stimulated (under fed conditions and stimulus like sucking) digestive fluids are described in detail in the publications by Oomen et al., 2003a and Versantvoort et al., 2005. Stimulated and non-stimulated digestive juices differ in pH, salt concentrations, and enzyme concentrations. The mixtures are rotated head-over-heels at 55 rpm and the whole process is performed at 37 °C. At the end of the digestion process the tubes are centrifuged for 5 min at 2750*g*, yielding the chyme (the supernatant) and the digested matrix (the pellet), and sampled to obtain information on the bioaccessibility of the contaminant. Samples can be taken from the saliva, stomach and chyme phase to obtain information on the bioaccessibility of the contaminant and its behaviour in the different compartments of the digestive tract.

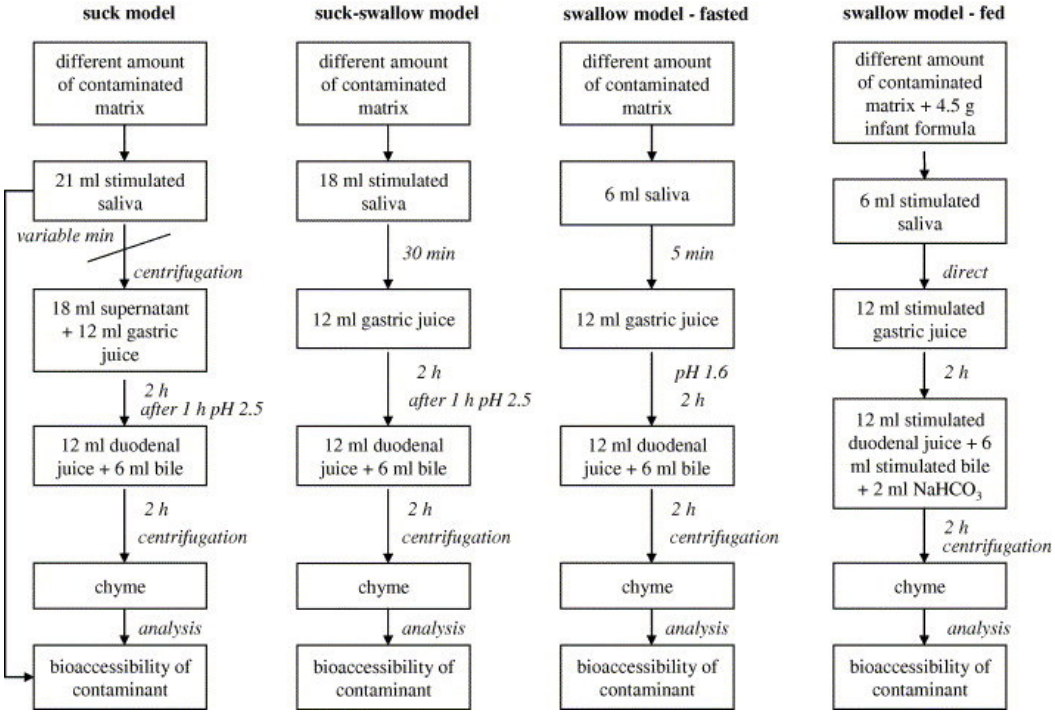

Fig. 2. Schematic representation of the suck, suck-swallow, and swallow under fasted and fed conditions in vitro digestion models.

2.2.1. Suck model

The suck model is applied to simulate sucking by a child on a consumer product (Oomen et al., 2003b). The suck time depends on the age of the child (at the age of 0.5–2 years children have the longest suck time), but also on the product (Bremmer and Van Veen, 2002 and Groot et al., 1998). The migration of a contaminant from its matrix into saliva simulant after a certain time based on mouthing duration can be measured with this model. It can either be assumed that all the contaminant that is released in the mouth is also available for absorption, in which case the model is terminated after the mouth phase, or that the contaminant can form aggregates that cannot be absorbed in the stomach or intestinal compartment. With the suck model, the contaminant that is released in the mouth during sucking (one compartment model) or the fraction that is available for absorption in the small intestine (three compartment model) can be investigated. Different amounts of matrix are introduced to 21 ml stimulated saliva and rotated for a variable time periods. The time period applied can be either a default value (30 min) or a period considered to be specifically appropriate for a certain product which is based on the product and the input of the risk assessor. The digestion tubes are centrifuged to remove the matrix and 18 ml of supernatant used for further incubation (the other 3 ml are used for analysis of the bioaccessibility in saliva). For the one compartment model, the sucking model is terminated after the saliva incubation. For the three compartment model, a volume of 12 ml gastric juice (pH 1.07 ± 0.07) is added to the saliva supernatant. The mixture is rotated for 1 h and the pH of the mixture is determined and, if necessary, set to 2.5 ± 0.1. Then, the mixture is rotated for another hour. Finally, 12 ml of duodenal juice (pH 7.8 ± 0.2) and 6 ml bile (pH 8.0 ± 0.2) are added simultaneously, and the pH of the chyme is determined and if necessary set to 6.5 ± 0.5. Then, the mixture is rotated for another 2 h. The digestion tubes are centrifuged and the supernatant is suitable for analysis.

2.2.2. Suck-swallow model

The suck and swallow model is applied to simulate mouthing and then ingestion of a certain consumer product (Oomen et al., 2003b). Thus, contrary to the three compartment suck model the matrix is ingested after sucking. The only modification is that the digestion starts by introducing 18 ml stimulated saliva to different amounts of matrix. This mixture is rotated head-over-heels for 30 min and then gastric juice is directly added without centrifuging. The rest of the procedure is the same as described for the three compartment suck model.

2.2.3. Swallow model under fasted conditions

The swallow model is applied to simulate ingestion of a certain consumer product under fasted conditions (Oomen et al., 2003b). It starts by introducing 6 ml saliva (pH 6.5 ± 0.2) to different amounts of matrix. This mixture is rotated for 5 min. Subsequently, 12 ml of gastric juice is added and the pH of the mixture of saliva and gastric juice is determined and, if necessary, directly set to 1.6 ± 0.1. The mixture is rotated for 2 h. Finally, 12 ml of duodenal juice and 6 ml bile are added simultaneously and the pH is determined and if necessary set to 6.0 ± 0.5. The mixture is rotated for another 2 h. The digestion tubes are centrifuged and the supernatant is used for analysis.

2.2.4. Swallow model under fed conditions

An in vitro digestion model simulating fed conditions has been developed to quantify the bioaccessibility under fed conditions (Versantvoort et al., 2004). The digestion starts by introducing different amounts of matrix to 6 ml stimulated saliva and 4.5 g infant food (product number 282, Olvarit (Nutricia®, The Netherlands), supplemented with 2 ml sunflower oil per 100 g). This infant food with sunflower oil represents the mean food intake for adults in The Netherlands for a cooked meal regarding macronutrients and caloric composition. It is based on the third Dutch National Food Consumption Survey from 1998 (Versantvoort et al., 2005). Immediately, 12 ml of stimulated gastric juice (pH 1.30 ± 0.02) is added and pH of the mixture is set to 2.5 ± 0.5. After 2 h of rotating, 12 ml of stimulated duodenal juice (pH 8.1 ± 0.2), 6 ml stimulated bile (pH 8.2 ± 0.2), and 2 ml sodium bicarbonate (84.7 g/l) are added simultaneously. The pH is set to 6.5 ± 0.5 and the mixture is rotated for another 2 h. Separation of chyme and pellet was obtained by centrifugation and the supernatant can be analysed to determine the bioaccessibility of the contaminant.

2.3. Comparison of the models

The main differences between the suck, suck-swallow, and swallow in vitro digestion model are the stomach pH and the composition of the digestive juices. The matrix may affect the pH in the stomach. However, under fasting conditions, the pH in the stomach is usually low and set at 2.5 ± 0.1 in the suck and suck-swallow model and to 1.6 ± 0.1 in the swallow model under fasted conditions (see Fig. 2). The pH in the gastric compartment of the swallow model under fasted conditions is lower, because less saliva is entering the gastric compartment in the swallow model (6 ml saliva instead of 18 ml). These pHs were chosen because an in vitro digestion without matrix results in this pH and because this pH falls in the range of pH values for fasting conditions (Charman et al., 1997).

2.4. Water extraction

One of the methods applied by Inspection Authorities for measuring a leaching rate is based on extraction with water (NEN-EN 71-3:1995/C1:2002 en, 2002). The in vitro digestion models were compared with water extraction. For the water extraction, the procedure was the same as described for the other models, except that water was used instead of the digestion fluids and the pH was not set. The latter was not done, because only water was used and therefore no physiological conditions apply.

2.5. Consumer products tested in the digestion models

Different contaminants from different consumer products were tested for their bioaccessibility in the in vitro digestion models. The different contaminants and consumer products were azo dyes in textile, lead in finger paint, chalk, and paint scraped from tops, phthalates in PVC disks, and benzoic acid in finger paint. The real-life case material and the PVC disks were provided by the Dutch Food and Consumer Product Safety Authority. For the bioaccessibility determination, samples were taken from saliva (100 μl), the stomach (100 μl), and chyme (900 μl), when applicable. Montana Soil 2711 and SRM paint were taken into account as quality control samples. They both contained lead (Montana Soil 1.162 mg/g soil and SRM paint 4.49 mg/g paint) and the amounts used were 0.04 and 0.4 g per digestion tube to overcome possible saturation of the digestion juices.

2.5.1. Lead from finger paint

Finger paint (toy store) was spiked with lead ($Pb(NO_3)_2$) to a concentration of 25 mg/g wet weight. The finger paint consisted 50% of water. The bioaccessibility of lead from finger paint was investigated using the suck, suck-swallow, and the swallow model under fasted conditions. The suck model was used as a one compartment model (only saliva) and the three compartment model (entire digestive tract). The swallow model used was slightly different from the model described in an earlier paragraph, namely the pH in the stomach was set after 1 h instead of immediately. All experiments were performed in sixfold and with 0.4 g matrix per digestion tube to investigate the inter and intra assay variation and comparison of the different models.

2.5.2. Lead from chalk

Sidewalk chalk (real-life case) contained 28 mg lead/g chalk. Spiked chalk (analytical grade) was prepared with $Pb(NO_3)_2$ in our laboratory with a concentration of 1.5 mg lead/g chalk. The bioaccessibility of lead from chalk was investigated using the suck, suck-swallow, and the swallow model under fasted conditions. The spiked chalk was also studied in the swallow model under fed conditions. The suck model was used as a one compartment model (spiked) and the three compartment model (spiked and real-life case). The swallow model used was slightly different from the model described earlier, namely the pH in the stomach was set after 1 h instead of immediately. An amount of 0.4 g matrix was used in the suck, suck-swallow and the swallow model under fasted conditions and in an additional experiment an amount of 0.01 and 0.4 g was studied in the swallow model under fasted conditions. All experiments were performed in sixfold for intra assay variation determination and comparison of the different models.

2.5.3. Lead from paint scraped of tops

The Dutch Food and Consumer Product Safety Authority had found that the paint of one specific kind of top (real-life case) released 1970 μg lead per g paint in HCl (pH 1.5) which is highly above the allowed release rate of 0.7 μg/g. The paint was scraped from the tops for bioaccessibility testing. The paint was found to contain 14.79 ± 0.36 mg lead/g which is also highly above the allowed level of 0.0035 mg/g (=3.5 mg/kg). In all situations, a swallow model was used as it was assumed that paint from tops will be directly ingested by children after the paint has been scraped of with their teeth and the sucking phase is of minor importance. Furthermore, it is not likely that sucking on paint with lead represents the worst case scenario because lead is mainly released under acidic conditions which was based on the findings of the experiments with finger paint and sidewalk chalk. The following variables were tested: fasted and fed conditions and water extraction. Different amounts of paint (0.01–0.40 g per digestion tube) were investigated for their influence on the bioaccessibility of lead. The experiments were performed in duplicate.

2.5.4. Phthalates (DINP) from PVC disks

The same DINP containing PVC disks as in previous studies by Könemann, 1998 and Simoneau et al., 2001 were used. In short, a batch of 3-mm thick PVC pads was prepared in a laboratory. The final sheet was composed of PVC (58.8%), di-isononylphthalate (Jayflex® DINP) (38.2%), epoxidised soybean oil (1.8%) and Ca/Zn stabiliser (1.2%). Disks were punched from the sheet with a diameter of 23 mm and the total area of a disk was approximately 10 cm^2. The disks were used after 5 years of storage in the dark at room temperature and therefore the total content of DINP was determined before use. The mean concentrations of DINP in the three disks was 40.3 ± 1.9%, which is similar to the reported initial concentration of 38.2% (Könemann, 1998).The suck model was used and was restricted to the saliva phase. The suck time was set at 15 min per suck event with 4 separate suck events and the bioaccessibility and leaching rate were determined. The experiments were performed in fourfold and repeated after 3 weeks.

2.5.5. Azo dyes (aniline, 2,4-toluenediamine, o-dianisidine) from textile

Three textiles (real-life case) containing different azo dyes were investigated. The concentration of azo dyes was characterised by the amount of amines that were determined after reduction of the dyes per kg textile. One piece contained 1.51 mg aniline per g textile, another piece 0.499 mg of 2,4-diaminotoluene per g textile, and the third contained 0.709 ± 0.059 mg of *o*-dianisidine per g textile. In all situations the suck model (1 compartment) was used as it was assumed that children suck on pieces of textile and do not ingest them. The following variables were tested: suck time of 7.5–60 min which is normal mouthing time for young children (Groot et al., 1998, Juberg et al., 2001 and Bremmer and Van Veen, 2002), multiple suck events (3 separate times), washed versus non-washed textile, and water extraction versus physiologically relevant conditions. The aim of varying the suck time is to be able to determine an extraction rate that can be implemented in the human exposure model ConsExpo (Van Veen, 2001). All experiments were performed in triplicate and with 0.04–0.4 g textile per digestion.

2.5.6. Benzoic acid from finger paint

Five samples of finger paint (real-life case) containing benzoic acid at levels exceeding the limit values (0.5%) were provided by the Dutch Food and Consumer Product Safety Authority. The concentrations of benzoic acid in the different finger paint samples were 5.9 mg/g in green paint, 7.4 mg/g in yellow paint, 6.1 mg/g in blue paint, 7.3 mg/g in red paint and 6.4 mg/g in orange paint. Furthermore, blank green finger paint was spiked with benzoic acid to concentrations of 2.5, 5, 10, 20 and 50 mg/g wet weight. The finger paint consisted 50% of water. In all situations, a swallow model with a saliva phase was used as it is assumed that finger paint is directly ingested and the sucking phase is of minor importance. The following variables were tested: fasted and fed conditions (5 min saliva phase instead of direct stomach fluid addition) and water extraction. All experiments were performed as a single experiment, except for blue and red finger paint under fasted conditions which were performed in duplicate. The basis for performing three colours in a single experiment and two in duplicate was based on the assumption that the standard deviation is equal for the different colours of finger paint on the one hand and practical limitations (maximum of 50 samples per day) on the other hand. Different amounts of real-life case finger paint (0.01–1.0 g per digestion tube) were investigated for their influence on the bioaccessibility of benzoic acid. For experiments with spiked finger paint 0.4 g per digestion tube was used.

2.6. Analysis of the contaminants

The analysis of lead was performed by the Laboratory of Environmental Monitoring and the analysis of DINP was performed by the Laboratory for Analytical Food and Residue Research, both of the National Institute for Public Health and the Environment. The other contaminants were measured by the Dutch Food and Consumer Product Safety Authority.

2.6.1. Lead analysis

In short, the samples were diluted to a volume of 9 ml with 0.1 M HNO_3. The amount of lead was measured by means of ICP-MS and Renium (50 ng/ml) was used as internal standard. The limit of quantification was 180 ng/ml in saliva and stomach fluid and 20 ng/ml in chyme.

2.6.2. Analysis of azo dyes

The analysis was performed by GC-MS after solvent extraction from the digestion fluid with dichloromethane. The correlation coefficient of the calibration curve was 0.997, with an average recovery of 112.7% and a relative standard deviation of 12.1%. The limit of quantification for aniline was 1.0 ng/ml of extraction fluid, for 2,4-toluenediamine 5.0 ng/ml; and for *o*-dianisidine 20 ng/ml.

2.6.3. Analysis of phthalates

Analysis of DINP in saliva simulant was performed according to Schakel (2000). The total volume of saliva simulant was extracted with 15 ml iso-octane to avoid adsorption losses. The iso-octane solutions were analysed by straight phase HPLC using a cyanopropyl column with iso-octane as mobile phase and UV detection at 225 nm. Calibration curves were linear over the range of 0.25–5 μg DINP/ml iso-octane. Recovery of DINP from saliva simulant was higher than 95%.

2.6.4. Benzoic acid analysis

The samples were diluted to a volume of 2.5 ml of which 2 ml was added to 40 ml extraction fluid (1.15 g/l $NaC_2H_3O_2$, 1.2% (v/v) acetic acid and 37% (v/v) methanol in water, pH of 4.5). The samples were placed in a shaking water bath at 80 °C until it became a clear liquid. Extraction fluid was added until a volume of 90 ml and it was left to cool down to room temperature. Then, the solution was placed in an ultrasound bath for 10 min. One ml Carrez I solution (106 g/l $K_4Fe(CN)_6 \cdot 3H_2O$), 1 ml Carrez II solution (220 g/l $Zn(C_2H_3O_2)_2$ and 2.9% acetic acid) and extraction fluid to a volume of 100 ml were added. Next, the solution was left for 10 min at room temperature and filtrated over filtration paper. The amount of benzoic acid was measured using HPLC analysis. The eluent comprised of 80% (v/v) acetate buffer (11.5 g/l $NaC_2H_3O_2$ and 11.5% acetic acid, pH 4.5) and 20% (v/v) acetonitrile. The eluent flow rate was 1.0 ml/min and the UV detection wavelength was set at 227 nm. The lower limit of quantification (LLQ) was 10 ng/ml in saliva and stomach fluid and 2 ng/ml in chyme.

2.7. Data analysis

The bioaccessibility can be calculated by dividing the amount in the digestive juice by the total amount in the contaminated sample before the digestion experiment. The amount in the digestive juice is measured by the method described above and corrected for the measured value in the blank sample and the total volume of digestive juice, sample and food. The results are expressed as means ± standard deviation (SD) or range, where applicable. Differences between the results were analysed by the Student *t* test for unpaired observations.

3. Results

An overview of the bioaccessibility results for the different matrices and contaminants is given per digestion model: Table 1, suck model; Table 2, suck-swallow model; Table 3, swallow model under fasted conditions, and Table 4, swallow model under fed conditions. In the following paragraphs a short overview of the results per matrix is given. More detailed information can be found in the reports 320102001/2003, 320102003/2004 and 320102004/2005 of the National Institute for Public Health and the Environment (Oomen et al., 2003b, Oomen et al., 2004 and Oomen et al., 2005).

Table 1.

Bioaccessibility from consumer products using the suck model

Contaminant	Matrix	Spiked or real-life case	Amount matrix	One or three compartment[a]	Bioaccessibility (%)
Lead	Finger paint	Spiked (25 mg/g)	0.4 g	1	9.2–13.2[b]
	Finger paint	Spiked (25 mg/g)	0.4 g	3	4.5–6.3[b]
	Chalk	Spiked (1.5 mg/g)	0.4 g	1	<1[b]
	Chalk	Spiked (1.5 mg/g)	0.4 g	3	<1[b]
	Chalk	Real-life case (28 mg/g)	0.4 g	3	0.1–0.3[b]
DINP	PVC disk	Spiked (≈40.3 mg/g)	10 cm^2	1	0.029–0.033[c,d]
Aniline	Textile	Real-life case (≈1.51 mg/g)	0.04 g	1	7.5–9.7[c]
			0.1 g		5.7–11.8[c]
			0.4 g		6.4[c]
	Washed textile	Real-life case (≈1.51 mg/g)	0.4 g	1	≈1[c]
2,4-Toluenediamine	Textile	Real-life case (≈0.50 mg/g)	0.04 g	1	3.1–8.1[c]
			0.1 g		3.3–9.8[c]
			0.4 g		5.2[c]
	Washed textile	Real-life case (≈0.50 mg/g)	0.4 g	1	3.9[c]
o-Dianisidine	Textile	Real-life case (≈0.71 mg/g)	0.04 g	1	0.37–0.45[c]
			0.1 g		0.38–0.66[c]
			0.4 g		0.14–0.24[c]
	Washed textile	Real-life case (≈0.71 mg/g)	0.4 g	1	0.17–0.45[c]

[a] The one compartment is the saliva phase and the three compartment is mouth, stomach and intestine.
[b] Report 320102001/2003 (Oomen et al., 2003b).
[c] Report 320102003/2004 (Oomen et al., 2004).
[d] Suck event 1 to 4.

Table 2.

Bioaccessibility from consumer products using the suck-swallow model

Contaminant	Matrix	Spiked or real-life case (mg/g)	Amount matrix (g)	Bioaccessibility (%)
Lead	Finger paint	Spiked (25)	0.4	15–19
	Chalk	Spiked (1.5)	0.4	0–3
	Chalk	Real-life case (28)	0.4	0.1

All data in the table is from report 320102001/2003 (Oomen et al., 2003b).

Table 3.

Bioaccessibility from consumer product using the swallow model under fasted conditions

Contaminant	Matrix	Spiked or real-life case (mg/g)	Amount matrix (g)	Bioaccessibility (%)
Lead	Finger paint[a]	Spiked (25)	0.4	40–62[c]
	Chalk[a]	Spiked (1.5)	0.4	0.5–2.7[c]
	Chalk[a]	Real-life case (28)	0.4	2.9–3.7[c]
	Chalk[a]	Real-life case (28)	0.01	30–61[d]
			0.04	18–27[d]
			0.1	9–13[d]
			0.4	2.5–4.9[d]
	Top paint[b]	Real-life case (14.8)	0.01	9.1 ± 1.8[e]
			0.04	10.7 ± 1.0[e]
			0.07	10.2 ± 0.7[e]
			0.10	9.1 ± 0.04[e]
			0.25	9.1 ± 1.0[e]
			0.40	9.1 ± 0.02[e]
Benzoic acid	Finger paint red[b]	Real-life case (7.3)	0.01	18.1 ± 2.3[e]
			0.04	34.9 ± 1.6[e]
			0.10	44.2 ± 2.6[e]
			0.25	62.9 ± 3.0[e]
			0.40	67.8 ± 0.8[e]
			1.00	66.9 ± 1.4[e]
	Finger paint blue[b]	Real-life case (6.1)	0.01	29.2 ± 8.8[e]
			0.04	33.3 ± 11.2[e]
			0.10	54.0 ± 5.4[e]
			0.25	71.6 ± 3.8[e]
			0.40	77.9 ± 1.1[e]
			1.00	79.3 ± 0.2[e]
	Finger paint green[b]	Spiked (2.5)	0.40	57.9[e]
		Spiked (5)	0.40	63.9[e]
		Spiked (10)	0.40	53.4[e]
		Spiked (20)	0.40	45.5[e]
		Spiked (50)	0.40	120.1[e]

[a] pH set to 1.6 ± 0.1 after 1 h.
[b] pH immediately set to 1.6 ± 0.1.
[c] Report 320102001/2003 (Oomen et al., 2003b).
[d] Report 320102003/2004 (Oomen et al., 2004).
[e] Report 320102004/2005 (Oomen et al., 2005).

Table 4.

Bioaccessibility from consumer product using the swallow model under fed conditions

Contaminant	Matrix	Spiked or real-life case (mg/g)	Amount tested (g)	Bioaccessibility (%)
Lead	Chalk	Spiked (1.5)	0.01	34–47[b]
			0.1	15–23[b]
	top paint	Real-life case (14.8)	0.01	3.5 ± 0.5[c]
			0.04	4.5 ± 0.3[c]
			0.07	4.9 ± 0.9[c]
			0.10	4.8 ± 0.5[c]
			0.25	5.3 ± 0.3[c]
			0.40	3.7 ± 0.03[c]
Benzoic acid	Finger paint red[a]	Real-life case (7.3)	0.01	107.5[c]
			0.04	78.7[c]
			0.10	80.8[c]
			0.25	82.4[c]
			0.40	81.2[c]
			1.00	79.8[c]
	Finger paint blue[b]	Real-life case (6.1)	0.01	9.9[c]
			0.04	11.5[c]
			0.10	19.6[c]
			0.25	66.4[c]
			0.40	79.1[c]
			1.00	97.1[c]
	Finger paint green[b]	Spiked (2.5)	0.40	83.4[c]
		Spiked (5)	0.40	88.4[c]
		Spiked (10)	0.40	88.2[c]
		Spiked (20)	0.40	74.2[c]
		Spiked (50)	0.40	86.5[c]

[a] 5 min incubation with saliva before adding the gastric juice.
[b] Report 320102003/2004 (Oomen et al., 2004).
[c] Report 320102004/2005 (Oomen et al., 2005).

3.1. Lead from finger paint

The bioaccessibility of spiked lead from finger paint was the highest using the swallow model under fasted conditions (40–62%; Table 3) compared to the suck (9.2–13.2%; Table 1) and the suck-swallow model (15–19%; Table 2). This indicates that for ingestion of lead contaminated finger paint, the swallow model under fasted conditions will result in the most realistic worst case scenario for risk assessment. This situation leads to the highest bioaccessibility and thus highest internal exposure and it might be lower than the value used in current risk assessment. The bioaccessibilities of lead in the one and three compartment suck model (Table 1) were 9.2–13.2 and 4.5–6.3%, respectively, indicating that some lead salts are probably precipitated in the gastrointestinal tract. However, since the one compartment model did not result in an underestimation of the bioaccessibility in the suck model, it could thus be used to study the exposure of a child sucking on a consumer product. The bioaccessibility in the stomach compartment was higher in all cases compared to the intestine for the suck-swallow and swallow model, indicating a pH dependent release of lead into the digestive juices (results not shown).

3.2. Lead from chalk

The bioaccessibility of spiked lead from chalk was the highest when using the swallow model (0.5–2.7%; Table 3) compared to the suck (<1%; Table 1) and the suck-swallow model (0–3%; Table 2). In line with the results for lead in finger paint, this indicates that the swallow model under fasted conditions will result in the most realistic worst case scenario for risk assessment. The bioaccessibilities from lead in the one and three compartment suck model (Table 1) were both below 1%. The sidewalk chalk (real-life case) gave comparable bioaccessibility results compared to the spiked material. The results were 0.1–0.3% in the suck model (Table 1), 0.1% in the suck-swallow model (Table 2), and 2.9–3.7% in the swallow model under fasted conditions (Table 3), indicating that the swallow model will result in the highest exposure. The bioaccessibility of lead from spiked and real-life case chalk was for the suck-swallow and swallow model higher in the stomach compartment compared to the intestine, indicating a pH depended release of lead into the digestive juices (results not shown). An amount dependent release of lead was observed for the sidewalk chalk, indicating a saturation of the digestive fluids.

3.3. Lead in paint scraped from tops

Under fed conditions (≈4%) the bioaccessibility of lead in chyme was lower than under fasted conditions (≈9.5%), but in both cases it was low. However, the fasted model should be used for risk assessment because this results in the highest exposure scenario. The different amounts of paint did not influence the bioaccessibility of lead, indicating that the model did not reach its limitations for solubility of lead in the digestive juices. The bioaccessibility of lead was higher in the stomach compartment compared to the intestine, indicating a pH dependent release of lead into the digestive juices (results not shown). The bioaccessibility of lead in water appeared to be very low (<3%) (results not shown). The bioaccessibility in water was not influenced by the amount of paint and the duration of extraction (5 min, 2 h and 4 h).

3.4. Phthalates (DINP) from PVC disks

The migration rate (±3.5 μg/min) of DINP from the PVC disks was similar for every time period sampled (single results not shown). The mean leaching rate of DINP from PVC disks was 0.3 μg/min cm^2 and the bioaccessibility was low (0.029–0.033%). The different suck events gave the same leaching rate and bioaccessibility. Therefore, only the mean results are shown. When the disks were used again after three weeks, the migration rate of DINP was the same (results not shown). The migration rate (1.8 ± 0.6 μg/min) and bioaccessibility (0.016–0.022%) of DINP in water were lower compared to saliva, indicating that water is not the right extraction fluid for DINP to estimate human exposure.

3.5. Azo dyes from textile

The three azo dyes showed different bioaccessibility results in the suck model. Aniline and 2,4-toluenediamine had almost the same bioaccessibility (5.7–11.8 and 3.1–9.8%, respectively), *o*-dianisidine had a lower bioaccessibility (0.14–0.66%). The release of the azo dyes was not dependent on the amount of textile in the model or the suck time (results not shown). The bioaccessibility of aniline after the suck event was approximately 8%, corresponding to a release of 120 μg aniline per g textile. When the same pieces of textile were digested a second or third time, the bioaccessibility decreased to 1–2% (12–24 μg/g). This suggests that part of the azo dye in textile is weakly bound. Although indicative due to the

small number of repeated sucking events, it can be assumed that probably less than 20% of aniline will be released from the textile by multiple sucking events. The bioaccessibility of 2,4-toluenediamine and *o*-dianisidine did not differ between the first, second, or third suck event with the same pieces of textile (results not shown). This suggests that the same amount of azo dye that produces the amine 2,4-toluenediamine and *o*-dianisidine after reduction was released from textile, irrespective of whether the piece of textile has been sucked on before or not. Washed textile resulted in a lower bioaccessibility for aniline, but not for *o*-dianisidine and 2,4-toluenediamine. The effect of water as extraction fluid was also investigated. The azo dyes aniline and *o*-dianisidine had the same bioaccessibility compared to stimulated saliva (results not shown). However, 2,4-toluenediamine showed a slight decrease in release in water from the textile compared to saliva (1.5–7.9%), however, this was not significant. It still indicates that water as extraction fluid, as described in EU legislation, is not always the correct extraction fluid to investigate the release of a contaminant during sucking of a child on a consumer product.

3.6. Benzoic acid from finger paint

Bioaccessibility of benzoic acid in the intestine at fasted conditions was between 23 and 83% for the different amount and colours of finger paint (results only shown for blue and red paint and not for the other colours). Bioaccessibility of benzoic acid tended to increase with increasing amounts of finger paint in the test tube. This could be due to the clean-up procedure of the intestinal fluid for analysis. A standard amount of benzoic acid could be lost during the clean-up, influencing the bioaccessibility of the lower amounts more than the higher amounts. Furthermore, the data show a tendency for higher bioaccessibility at fed conditions than at fasted conditions. Water extraction and the digestion models showed comparable bioaccessibility values (results not shown), indicating that for benzoic acid from finger paint water could be used as extraction fluid. However, this conclusion cannot be extrapolated for other contaminants from finger paint or benzoic acid from other matrices. Finger paint spiked with benzoic acid showed the same results as real-life case finger paint for the different contamination levels under fasted and fed conditions and also after water extraction.

3.7. Variation

The intra and inter day variations were low (<10%) when the matrices were homogeneously contaminated, e.g., lead in paint scraped from top. However, higher intra variations (>25%) were observed for inhomogeneous samples, e.g., azo dyes from textile. The inter day variation was somewhat lower (≈20%) (Oomen et al., 2003b).

4. Discussion and conclusions

Determination of the internal exposure is a good approach to improve the risk assessment in cases where a first tier risk assessment indicated a potential risk. Therefore, a simple, fast and relatively inexpensive in vitro model based on human physiology was developed to predict a more realistic internal exposure to a contaminant after oral exposure to a consumer product.

All the in vitro digestion models developed in our laboratory, allow simulation of the release of a contaminant from a matrix during transit in the gastrointestinal tract (bioaccessibility). The digestion models do not take the large intestine into account, because absorption of compounds mainly takes place in the small intestine. Therefore only the bioaccessibility determined in the chyme of the small

intestine is relevant for risk assessment. However, based on the absorption site other compartments (mouth or stomach) can be chosen.

To our knowledge, simulation of the different exposure scenario's sucking and/or swallowing have not been performed by others with experimental models in the risk assessment of toys or other consumer products. In the field of soil contamination and pharmacology other in vitro digestion models are used (Oomen et al., 2002). However to our knowledge, these models have not been used to investigate the bioaccessibility of contaminants from consumer products after swallowing. Until now, these models have not been adapted to simulate the suck and suck-swallow behaviour of children. Therefore, it was important that an in vitro digestion model was developed for consumer products simulating sucking and/or swallowing.

Validation of the developed in vitro digestion models for consumer product is difficult, because human in vivo data from consumer products with contaminants are scarce. Only, the results of the phthalate bioaccessibility from PVC disks with the suck model could be compared with human in vivo release data. The results were similar (Oomen et al., 2004). The developed in vitro digestion models for food and soil could be partially validated by comparing bioaccessibility with human in vivo bioavailability data or with animal data. Aflatoxin B1 and ochratoxin A in animal feed was used to validate the food in vitro model and lead and arsenic release from soil to validate the soil model. These in vitro bioaccessibilities were in accordance with the in vivo bioavailability (Oomen et al., 2003b, Versantvoort et al., 2005 and Oomen et al., in press). Validation check will be continued were possible.

Due to the between-day-variation which is caused by the heterogenicity of the contamination, it is advised to test potential differences between matrices or other variables, e.g., amounts, in one experiment and not in different experiments. Furthermore, it is recommended to test the matrix with 1 or 2 experiments and 3 to 4 replicates, including a relevant reference matrix. The absolute bioaccessibility will become more reliable when the experiment has several repeats. The CSTEE also does not accept the use of one representative to determine the release from toys because of their heterogeneous nature (CSTEE, 2004). The highest bioaccessibility results can be used to calculate the highest realistic exposure of a child to the contaminant in the consumer product.

The in vitro digestion models allow for simultaneous assessment of the bioaccessibility of dozens of samples within an experimental series, so that physiologically and exposure relevant variables can be included at low costs and within a limited amount of time. Variation in suck time, multiple suck events, variation of the amount of matrix, and the different digestion conditions under fasted and fed conditions are of importance when assessing the bioaccessibility during sucking and/or swallowing. We recommend to test different amount of matrix in order to avoid saturation of the digestive fluids. Such saturation could lead to underestimation of the actual bioaccessibility. The experimental design of the in vitro digestion model is adjustable to input of the risk assessors for specific exposure scenarios, so that the outcome of the model is useful for realistic risk assessment for children. The consumer product in vitro digestion model can be applied to a limited time frame, so that it can be applied when immediate information on the bioaccessibility is needed in case of an ad hoc question.

A study by the European Committee for the Standardisation (CEN) showed that for some chemicals in a PVC disk (acetyltributyl citrate, phenol, naphthalene, isophorone, tricresylphophate, xylene, benzylalcohol) water could be used instead

of saliva simulant for mobilisation of the chemicals, although reproducibility between laboratories was poor in some cases (Strikwerda, 2002 and Hillersborg, 2002). However, the azo dye 2,4-toluenediamine and the phthalate DINP showed a decrease in release from their matrix in water compared to saliva in our laboratory. This indicates that water is not always the correct extraction fluid to investigate the release of a contaminant during sucking by a child on a consumer product. We therefore strongly recommend to use saliva to study the release of a contaminant from a consumer product.

Mathematical consumer exposure models such as ConsExpo are currently being used in risk assessment to estimate internal exposure after ingestion, inhalation or dermal contact with a contaminated product (Van Veen, 2001). In many cases, too little information is available for describing the migration of contaminants from the consumer product for a reliable exposure assessment. The suck model can be used to refine the default values for release rate and the absorbed fraction of the "mouthing" scenario. The suck-swallow and the swallow models can be used to refine the default value for the absorbed fraction of the "hand-mouth contact" scenario. Subsequently, outcomes of the consumer exposure models can be directly applied by risk assessors for a specific ad hoc situation and lead to a realistic value for internal exposure in contrast to the current extraction fluids.

In conclusion, the in vitro digestion models are a fast, simple and relatively inexpensive method to determine the realistic worst case internal exposure for a more accurate risk assessment without the use of animals. The experimental design can be completely adjusted to the exposure scenarios formulated by the risk assessor, which are geared to real-life cases. However, the oral exposure route should be considered relevant in relation to other possible exposure routes such as inhalation or dermal exposure. The results of the model can be applied in the exposure models such as ConsExpo, resulting in an experimentally based exposure assessment. It is advised to apply the presently developed consumer product in vitro digestion model especially in ad hoc situations, because this could lead to an efficient and scientifically improved procedure in risk assessment. The following procedure is recommended: step (1) risk assessors set up exposure scenario's which are relevant for a specific contaminated matrix and give information about the relevance of oral exposure, step (2) in order to avoid unnecessary extrapolation steps, these exposure scenarios will be simulated in the model, step (3) outcomes of the model will be used in a mathematical exposure model (e.g., ConsExpo), and step (4) outcomes of the digestion model/exposure model will be applied by risk assessors for that specific ad hoc situation. Relevant for upholders is that a simple and fast performable in vitro digestion model was developed, which has added value for the present risk assessment in cases of ad hoc situations.

Acknowledgments

This project was supported by the Food and Consumer Product Safety Authority, The Netherlands. We thank Erwin van de Kamp and Klaas van Twillert of the Laboratory for Food and Residue Analyses of the National Institute for Public Health and the Environment for performing the in vitro digestion experiments. We also thank Petra Krystek and others of the Laboratory of Environmental Monitoring of the National Institute for Public Health and the Environment for performing the metal analysis. Furthermore, the analysis of the samples containing azo dyes and benzoic acid was kindly performed by the Dutch Food and Consumer Product Safety Authority in Groningen, The Netherlands.

References

Bremmer and Van Veen, 2002 Bremmer, H.J., Van Veen, M.P., 2002. Report No. 612810012. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/612810012.pdf>.

Charman et al., 1997 W.N. Charman, C.J.H. Porter, S. Mithani and J.B. Dressman, Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH, *J. Pharm. Sci.* **86** (1997), pp. 269–282.

Commission Directive COM 2005 (143), 2005 Commission Directive COM 2005 (143), 2005. Restrictions on the marketing and use of certain dangerous substances and preparations (phthalates) and amending council Directive 88/378/EEC on the approximation of the laws of the Member States concerning the safety of toys (COM (1999) 577—C5—0276/1999—1999/0238 (COD)).

Commission Directive 1999/815/EC, 1999 Commission Directive 1999/815/EC, 1999. 1999/815/EC: Commission Decision of 7 December 1999 adopting measures prohibiting the placing on the market of toys and childcare articles intended to be placed in the mouth by children under three years of age made of soft PVC containing one or more of the substances di-iso-nonyl phthalate (DINP), di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), di-iso-decyl phthalate (DIDP), di-*n*-octyl phthalate (DNOP), and butylbenzyl phthalate (BBP). L315, pp. 46–49.

Commission Directive 93/11/EEC, 1993 Commission Directive 93/11/EEC, 1993. Commission Directive 93/11/EEC of 15 March 1993 concerning the release of the *N*-nitrosamines and *N*-nitrosatable substances from elastomer or rubber teats and soothers. L093, pp. 37–38.

Commission Directive 88/378/EEC, 1988 Commission Directive 88/378/EEC, 1988. Council Directive 88/378/EEC of 3 May 1988 on the approximation of the laws of the Member States concerning the safety of toys. L187, pp. 1–13.

CSTEE, 2004 CSTEE, 2004. Opinion on Assessment of the European Committee for Standardisation (CEN) on Methods Development 43rd Plenary Meeting, 28 May.

Groot et al., 1998 Groot, M.E., Lekkerkerk, M.C., Steenbekkers, L.P.A., 1998. Mouthing behaviour of young children: An observational study. Household and Consumer Studies, Agricultural University, Wageningen.

Hillersborg, 2002 Hillersborg, A.S., 2002. CEN/TC 52 N851. CEN/TC 52—Safety of toys, Denmark.

Juberg et al., 2001 D.R. Juberg, K. Alfano, R.J. Coughlin and K.M. Thompson, An observational study of object mouthing behavior by young children, *Pediatrics* **107** (2001), pp. 135–142.

Könemann, 1998 Könemann, W.H., 1998. Phthalate release from soft PVC baby toys. Report from the Dutch Consensus Group. Report No. 613320002. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/613320002.pdf>.

NEN-EN 71-9:2003 Ontw. en, 2003 NEN-EN 71-9:2003 Ontw. en, 2003. Safety of toys—Part 9: Organic Chemical Compounds—Requirements (Referred to as Part Y).

NEN-EN 71-7:2002 (en), 2002 NEN-EN 71-7:2002 (en), 2002. Safety of toys—Part 7: Finger paints—Requirements and test methods.

NEN-EN 71-3:1995/C1:2002 en, 2002 NEN-EN 71-3:1995/C1:2002 en, 2002. Safety of toys—Part 3: Migration of certain elements.

Oomen et al., in press Oomen, A.G., Brandon, E.F.A., Swartjes, F.A., Sips, A.J.A.M., (in press). How can information on oral bioavailability improve human health risk assessment for lead-contaminated soils? Implementation and scientific basis. Report no. 711701042, Bilthoven, the Netherlands: National Institute for Public Health and the Environment.

Oomen et al., 2002 A.G. Oomen, A. Hack, M. Minekus, E. Zeijdner, C. Cornelis, G. Schoeters, W. Verstraete, T. van deWiele, J. Wragg, C.J.M. Rompelberg, A.J.A.M. Sips and J.V. Wijnen, Comparison

of five in vitro digestion models to study the bioaccessibility of soil contaminants, *Environ. Sci. Technol.* **36** (2002), pp. 3326–3334.

Oomen et al., 2005 Oomen, A.G., Rompelberg, C.J.M., Brandon, E.F.A., Van de Kamp, E., Duits, M.R., Versantvoort ,C.H.M., van Engelen, J.G.M., Sips, A.J.A.M., 2005. Consumer product in vitro digestion model: bioaccessibility of contaminants from toys and application in risk assessment. Report No. 320102004. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: < http://www.rivm.nl/bibliotheek/rapporten/320102004.pdf>.

Oomen et al., 2003a A.G. Oomen, C.J.M. Rompelberg, M.A. Bruil, C.J.G. Dobbe, D.P.K.H. Pereboom and A.J.A.M. Sips, Development of an in vitro digestion model for estimation of bioaccessibility of soil contaminants, *Arch. Environ. Contam. Toxicol.* **44** (2003), pp. 281–287.

Oomen et al., 2003b Oomen, A.G., Van Twillert, K., Hofhuis, M.F.A., Rompelberg, C.J.M., Versantvoort, C.H.M., 2003b. Development and suitability of in vitro digestion models in assessing bioaccessibility of lead from toy matrices. Report No. 320102001. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/320102001.pdf>.

Oomen et al., 2004 Oomen, A.G., Versantvoort, C.H.M., Duits, M.R., Van de Kamp, E., Van Twillert, K., 2004. Application of in vitro digestion models to assess release of lead and phthalate from toy matrices and azo dyes from textile. Report No. 320102003. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/320102003.pdf>.

Schakel, 2000 Schakel, D.J., 2000. De Ware(n) Chemicus. Inspectorate for Health Protection and Veterinary Public Health, Groningen, The Netherlands.

Simoneau et al., 2001 Simoneau, C., Geiss, H., Roncari, A., Zocchi, P., Hannaert, P., 2001. European Commission, DG—Joint Research Centre, Food Products Unit, Institute for Health and Consumer Protection, Ispra, Italy.

Strikwerda, 2002 Strikwerda, K., 2002. N 164 REV 1. CEN/TC 52/WG 9/TG2, Groningen, The Netherlands.

Van Veen, 2001 Van Veen, M.P., 2001. CONSEXPO 3.0; Consumer exposure and uptake models. Report No. 612810011. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/612810011.pdf>.

Versantvoort et al., 2005 C.H. Versantvoort, A.G. Oomen, E. Van de Kamp, C.J. Rompelberg and A.J. Sips, Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food, *Food Chem Toxicol* **43** (2005), pp. 31–40.

Versantvoort et al., 2004 Versantvoort, C.H.M., Van de Kamp, E., Rompelberg, C.J.M., 2004. Development of an in vitro digestion model to determine the bioaccessibility of contaminants from food. Report No. 320102002. National Institute for Public Health and the Environment, Bilthoven, The Netherlands. Available at: <http://www.rivm.nl/bibliotheek/rapporten/320102002.pdf>.

Wienk et al., 1999 K.J.H. Wienk, J.J.M. Marx and A.C. Beynen, The concept of iron bioavailability and its assessment, *Eur. J. Nutr.* **38** (1999), pp. 51–75.