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Application of *in vitro* digestion models to assess release of lead and phthalate from toy matrices and azo dyes from textile

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Abstract

The present project aims at the development of a simple and fast method to simulate the release of a compound from a certain toy matrix if a child sucks on the toy and/or ingests it. Only contaminants released from their matrix can reach the blood stream (i.e. internal exposure) and can exert toxicity. To simulate the release of contaminants from toys in the gastro-intestinal tract in a simple but physiological manner, three physiologically based in vitro digestion models have been developed in this project (RIVM report 320102001). By using only this released fraction of the contaminant for exposure assessment, the risk assessment can be refined and risks will be less easily overestimated.
The present report describes the application of the in vitro digestion models to several cases. The following cases were investigated: 1) the effect of the amount of matrix on the bioaccessibility of lead from chalk and paint flakes in the stomach and intestinal compartment, 2) release of a phthalate from PVC disks into saliva simulant, and 3) release of azo dyes from textile into saliva simulant. The release into digestive juice was in all cases considerably less than 100%, indicating that children are probably only exposed to a fraction of contaminants in the tested toy matrices. The release rate of phthalate from PVC disks was comparable to the release rate of phthalate from the same disks into saliva in a human volunteer study. This indicates that the in vitro digestion models are promising tools for exposure assessment of contaminants in toys.

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Contents

Samenvatting

Het doel van het huidige project is het ontwikkelen van een eenvoudige en snelle methode om het vrijkomen van stoffen uit een bepaalde speelgoedmatrix te simuleren als een kind op het speelgoed sabbelt of het inslikt. Alleen de contaminanten die worden vrijgemaakt van hun matrix in het maagdarmkanaal kunnen de bloedbaan bereiken (oftewel bijdragen aan interne blootstelling) en toxiciteit veroorzaken. Om het vrijkomen van contaminanten uit speelgoed in het maagdarmkanaal te simuleren op eenvoudige doch fysiologische wijze, zijn in het huidige project drie fysiologisch gebaseerde in vitro digestiemodellen ontwikkeld. Het huidige rapport beschrijft de toepassing van de in vitro digestiemodellen aan verschillende (praktijk)voorbeelden. De volgende (praktijk)voorbeelden zijn bestudeerd: 1) het effect van de hoeveelheid matrix op het vrijkomen van lood uit stoepkrijt of verfschilfers in de maag- en darmfase, 2) het vrijkomen van ftalaat uit PVC schijfjes in speekselsimulant, en 3) het vrijkomen van azo-kleurstoffen uit textiel in speekselsimulant. De resultaten van de verschillende studies worden hieronder behandeld.

Het effect van de hoeveelheid matrix op het vrijkomen van lood (Pb) uit stoepkrijt en verfschilfers in de maag- en darmfase staat beschreven in Hoofdstuk 2. Verschillende vast-vloeistof verhoudingen zijn bestudeerd om te simuleren dat eenmalig een grote hoeveelheid matrix wordt ingeslikt, of te simuleren dat een kleine hoeveelheid matrix per tijdseenheid wordt ingeslikt door hand-mond gedrag. De hoeveelheid matrix bleek een substantieel effect te kunnen hebben op het vrijkomen van Pb uit speelgoed. Zo was bijvoorbeeld ongeveer 50% Pb vrijgemaakt in de darmfase uit een kleine hoeveelheid stoepkrijt (vast-vloeistof ratio 1:1800), en ongeveer 4% uit een grote hoeveelheid stoepkrijt (vast-vloeistof ratio 1:45). Dit betekent dat de hoeveelheid speelgoed in acht moet worden genomen bij toepassing van de resultaten van het digestiemodel in blootstellingsschatting.
Er werd maximaal 52% van het Pb vrijgemaakt uit verfschilfers en 53% uit stoepkrijt. Dit suggereert dat een groot deel, ten minste 47-48%, van Pb in deze matrices niet bijdraagt aan de interne blootstelling als een kind deze gecontamineerde matrices inslikt. Daarnaast bleek dat de in vitro digestieprocedure goed reproduceerbaar was op basis van de resultaten van verschillende testdagen en vergelijking met eerder verkregen waarden.

Hoofdstuk 3 beschrijft het vrijkomen van ftalaat (di-isononylftalaat = DINP) uit PVC-schijfjes in speekselsimulant, en de vergelijking met andere vitro en vivo data. Migratie van DINP uit PVC-schijfjes was lineair in de tijd en bedroeg 3,3 μg/min, wat overeenkomt met 0,03% na 60 minuten sabbelen. Migratie van DINP uit de PVC-schijfjes in speekselsimulant van het RIVM (3,3 ± 0,5 μg/min) was ongeveer tweemaal zo hoog als migratie in speekselsimulant volgens het Joint Research Centre (JRC) zonder mucine (1,4 ± 0,4 μg/min), of in water (1,8 ± 0,6 μg/min). Deze drie migratiesnelheden waren in dezelfde ordegrootte als de gemiddelde migratiesnelheid van DINP uit de PVC-schijfjes in een humane vrijwilligersstudie (1,4 μg/min, variërend tussen 0,3 en 8,3 μg/min). Dit betekent dat het in vitro digestiemodel een geschikt instrument lijkt te zijn om de gemiddelde blootstelling te schatten van kinderen aan mogelijk schadelijke stoffen door sabbelen op speelgoed.

Het vrijkomen van azo-kleurstoffen uit textiel in speekselsimulant wordt bestudeerd in hoofdstuk 4. Omdat de bepaling van azo-kleurstoffen in (textiel)producten gebaseerd is op het aantonen van aromatische amines na reductie van de azo-kleurstoffen, zijn de resultaten uitgedrukt op basis van de aromatische amines. Slechts een klein deel van de azo-kleurstoffen kwam vrij uit textiel tijdens sabbelen: ongeveer 8% aniline, 7% 2,4-tolueendiamine en 0,6%

o-dianisidine werd gevonden in speekselsimulant. Dit betekent dat kinderen aan een fractie (respectivelijk 8%, 7% en 0,6%) van de totale hoeveelheid amine worden blootgesteld als ze *één* keer op de textielen sabbelen. De verschillende aromatische amines vertonen verschillend gedrag. Voor aniline kan worden afgeleid dat maximaal 20% vrijkomt bij meerdere malen sabbelen op het bestudeerde textiel, en dit percentage neemt aanzienlijk af na wassen van het textiel. Voor het textiel met 2,4-tolueendiamine moet onderscheid worden gemaakt tussen eenmaal sabbelen (7% vrijlating) en meerdere malen sabbelen (tot 100% vrijlating bij veelvuldig sabbelen). Het speeksel raakte waarschijnlijk verzadigd met de azo-kleurstof die de amine o-dianisidine bevat, in welk geval een extractiesnelheid van 72 ng o-dianisidine per minuut sabbelen kan worden afgeleid op basis van een maximale speekselingestiesnelheid van 4 ml/min in kinderen.
De resultaten van 2,4-tolueendiamine en o-dianisidine zijn vergeleken met aannames voor de migratie van deze amines uit textielproducten in beoordeling van kankerrisico. Vergelijkbare waarden voor de migratie van de amines zouden worden afgeleid voor de huidige producten als voor de beoordeelde producten. Dit komt doordat in de huidige experimenten met slechts 3-maal sabbelen op hetzelfde textiel geen afname in het vrijkomen kon worden bepaald (2,4-tolueendiamine), en omdat migratie in de kankerrisicobeoordeling was gebaseerd op een experimenteel bepaalde migratie in zweetsimulant, dat een vergelijkbare resultaat gaf als speekselsimulant (o-dianisidine). Er wordt geadviseerd om veelvuldig sabbelen beter te simuleren (~ 10-15 maal) om de uitloging van azo-kleurstof bij langdurig gebruik van textiel beter te kunnen schatten.

De resultaten van de experimenten met de in vitro digestiemodellen kunnen worden gebruikt om de standaardwaarden voor uitloging in de “fact sheet” speelgoed in ConsExpo te kunnen verfijnen. ConsExpo is een software pakket dat een aantal voorspellende modellen implementeert waarmee humane blootstelling aan stoffen in consumentenproducten kan worden geschat. Echter, omdat het vrijkomen van stoffen sterk afhankelijk bleek te zijn van de matrix en de stof zelf, wordt geadviseerd om het vrijkomen van stoffen experimenteel te bepalen voor nieuwe combinaties van matrix en stof. Als meer informatie beschikbaar komt kunnen relaties worden gelegd tussen vrijkomen van de stof en matrix eigenschappen, welke geïmplementeerd kunnen worden in de fact sheet van ConsExpo.

De huidige (praktijk)voorbeelden laten zien dat resultaten van experimenten met de in vitro digestiemodellen erg bruikbaar kunnen zijn in blootstellingsschatting en risicobeoordeling. Een eerste validatie van de modellen is verricht op basis van de mobilisatie van DINP uit PVC schijfjes naar speekselsimulant, en bleek bevredigend. Het kan daarom worden geconcludeerd dat de in vitro digestiemodellen veelbelovende instrumenten zijn voor betere blootstellingsschatting van contaminanten in speelgoed. De digestiemodellen bestaan uit een mond, maag- en darmcompartiment om sabbelen en inslikken van gecontamineerd speelgoed na te bootsen. De huidige validatie omvat alleen de mondfase. Daarom wordt geadviseerd verder validatie te verrichten van de modellen welke tevens de maag- en darmfase beslaat.

Summary

The present project aims at the development of a simple and fast method to simulate the release of a compound from a certain toy matrix if a child sucks on the toy and/or ingests it. Only contaminants released from their matrix in the gastrointestinal tract can reach the blood stream (i.e. internal exposure) and can exert toxicity. To simulate the release of contaminants from toys in the gastro-intestinal tract in a simple but physiological manner, three physiologically based in vitro digestion models have been developed in this project. The present report describes the application of the in vitro digestion models to several cases. The following cases were investigated: 1) the effect of the amount of matrix on the release of lead from chalk and paint flakes in the stomach and intestinal phase, 2) release of a phthalate from PVC disks into saliva simulant, and 3) release of azo dyes from textile into saliva simulant. The results of the case studies are addressed below.

The effect of the amount of matrix on the release of lead (Pb) from chalk for exterior use (NL: stoepkrijt) and paint flakes in the stomach and intestinal phase is described in Chapter 2. Different solid-to-fluid ratios were tested in order to simulate ingestion of a large amount of matrix during a single event, or to simulate ingestion of a small amount of matrix via hand-to-mouth behaviour. It appeared that the amount of matrix can have substantial effect on the release of Pb from toy. For example, about 50% of Pb was released from a small amount of chalk in the intestinal compartment (solid-to-fluid ratio 1:1800), and about 4% from a large amount of chalk (solid-to-fluid ratio 1:45). This indicates that the amount of toy matrix should be considered when using the in vitro digestion model and when applying the results of the digestion model in exposure assessment.
At maximum 52% of Pb was released from paint and 53% from chalk. This suggests that a considerable fraction, at least 47-48%, of the Pb in chalk and paint does not contribute to internal exposure when a child ingests these contaminated matrices. Furthermore, it appeared that the in vitro digestion procedure was well reproducible, as the release of Pb from chalk and paint on three different test days was very similar and in good agreement with values obtained before.

In Chapter 3, release of phthalate (di-isononylphthalate = DINP) from PVC disks into saliva simulant was studied and compared to other vitro and vivo data. Migration of DINP from PVC disks was linear in time and amounted 3.3 µg/min, corresponding to a release of 0.03% after 60 min of sucking. Migration of DINP from the PVC disks into saliva simulant of RIVM (3.3 ± 0.5 µg/min) was ~2-fold higher as migration into saliva simulant according to the Joint Research Centre (JRC) without mucin (1.4 ± 0.4 µg/min), or into water (1.8 ± 0.6 µg/min). These three migration rates were in the same order of magnitude as the average DINP release into saliva of human volunteers (1.4 µg/min, range 0.3-8.3 µg/min). Hence, the *in vitro* digestion model seems to be a suitable tool to estimate the average exposure of children to potentially harmful substances by mouthing toys and childcare articles.

The release of azo dyes from textile into saliva simulant is investigated in Chapter 4. Since determination of azo dyes in (textile) products is based on detection of aromatic amines that are obtained after reduction of the azo dyes, release figures refer to the aromatic amines. Release of the azo dyes from the textiles was low, i.e. approximately 8% aniline,

7% 2,4-toluenediamine, and 0.6% o-dianisidine was found in saliva simulant. This indicates that children are exposed to a fraction (respectively 8%, 7% or 0.6%) of the total amount of amine that can be obtained after reduction of the azo dye, when sucking *once* on the textile. Different patterns in release were observed for the three aromatic amines. For aniline, a maximum percentage can be estimated that can be released from the tested textile by multiple sucking events, which amounts 20% for untreated textile and is decreased considerably after washing the textile. For the textile containing 2,4-toluenediamine a distinction should be made between a single suck event (7% release), and multiple suck events (up to 100% released for a large number of suck events). The saliva simulant got probably saturated with the azo dye containing the amine o-dianisidine, in which case an extraction rate of 72 ng o-dianisidine per minute during sucking can be calculated based on a maximum saliva flow rate in children of 4 ml/min.
The results of 2,4-toluenediamine and o-dianisidine were compared with assumptions on the migration out of textile products made in the cancer risk assessment of these amines. Similar values for migration of the amines out of the textile products would have been derived for the present products as for the products in cancer risk assessment. This was because in the present experiments with only 3 repeated sucking events no decrease in release after sustained use could be derived (2,4-toluenediamine), and because the migration in the cancer risk assessment was based on experimentally determined migration into sweat simulant, which was similar to release into saliva simulant (o-dianisidine). It is recommended to simulate more suck events (~ 10-15 times) for better assessment of the release of azo dyes after sustained use of the textiles.

The results of experiments with the in vitro digestion models can be used to refine the default parameters for leaching in the fact sheet toys of ConsExpo. ConsExpo is a software tool that implements a set of predictive models to assess human exposure to chemicals in consumer products. However, as release appeared to be highly dependent on the contaminant and matrix, experimental determination of the release is recommended for new combinations of matrix and contaminant. When more information of a compound from various matrices becomes available, relationships between release of the compound and matrix characteristics can be made, which can be implemented in the fact sheets of ConsExpo.

The present case studies indicate that results of experiments with in vitro digestion models can be very useful in exposure c.q. risk assessment. A first validation of the models was performed with the mobilisation of DINP from PVC disks into saliva simulant, and found satisfactory. Hence, it was concluded that the in vitro digestion models are promising tools for exposure assessment of contaminants in toys. The digestion models consist of a mouth, stomach and intestinal compartment to simulate ingestion of contaminated toy matrices. The present validation comprises the mouth phase only. Therefore, it is recommended to perform further validation of the models including the gastric and intestinal compartments.

1. Introduction

Children can be orally exposed to compounds released from toy (parts) by chewing, sucking and ingestion. These compounds (contaminants) may cause adverse effects. The type of matrix (chalk, paint, teething ring, textile etc) and physicochemical properties of the contaminant may have profound influence on the release of contaminants in the gastrointestinal tract. Only the contaminants released from their matrix can reach the blood stream (i.e. internal exposure) and can exert toxicity. In present risk assessment, the release of contaminant from toy is assumed to be 100%, or is determined under non-physiological conditions. As a consequence, it is reasonable to assume that the risk that is calculated for children due to exposure to contaminants in toys, is overestimated. The aim of the present project is to develop a simple and fast method to estimate the amount of contaminant that can be released from toy if a child sucks on it or ingests (parts of) the toy. To that end, three physiologically based in vitro digestion models have been developed that can assess the release of contaminants from toy into digestive juices. These models simulate the following situations 1) sucking on toy (*suck model*), 2) sucking on toy in combination with swallowing of the toy matrix (*suck-swallow model*), and 3) swallowing of toy matrix without a sucking phase (*swallow model*). The development of these in vitro digestion models is described by Oomen et al. (Oomen et al., 2003). Release of contaminants from toy into the digestive juice is referred to as *bioaccessibility*.

This report describes application of the in vitro digestion models three case studies. The following cases were investigated:

The effect of mouthing behaviour on the bioaccessibility of lead (Pb) from toy matrices. Two mouthing behaviours that lead to very different amounts of toy matrices ingested are hand-to-mouth behaviour (licking/sucking) versus single ingestion. This different mouthing behaviour may give rise to different concentrations of matrix in digestive fluid, i.e. different solid-to-fluid ratios. In **Chapter 2**, it is studied whether the solid-to-fluid ratio affects the bioaccessibility of Pb from paint flakes and chalk for exterior use (NL: stoepkrijt) in the gastric and intestinal compartment.

In **Chapter 3**, bioaccessibility of phthalate (DINP) from PVC disks in the mouth phase is studied. Previously, experiments with humans have been performed with the same PVC disks (Simoneau et al., 2001; Könemann, 1998). Furthermore, an interlaboratory comparison on the migration of DINP into saliva simulant has been co-ordinated by the Joint Research Centre (JRC) (Simoneau et al., 2001; Könemann, 1998). Therefore, the release of DINP from the PVC disks in saliva simulant with the in vitro digestion model will be compared with the results obtained in vivo as a validation of the in vitro digestion model.

The release of azo dyes from textile by sucking is studied in **Chapter 4**. Only the saliva phase is simulated because azo dyes are reduced in the human intestine by bacteria to aromatic amines, which are well absorbed. Therefore, it is assumed that all dye that is released from the textile during sucking is absorbed. The results are discussed in light of application in the human exposure model ConsExpo (Van Veen, 2001), and in light of assessment of the cancer risk made by Zeilmaker et al. (Zeilmaker et al., 2000; Zeilmaker et al., 1999).

2. Hand-to-mouth behaviour versus ingestion

2.1 Introduction

In applying the in vitro digestion models, a toy matrix is added to a certain volume of digestive juices. The amount of toy matrix relative to the volume of digestive juices (solid-to-fluid ratio) may affect the bioaccessibility. In the present document it is studied whether the fraction of contaminant that is released from toy, i.e. bioaccessibility, is different after ingestion of a small amount of toy matrix compared to a large amount. Solid-to-fluid ratios are chosen that are representative for the in vivo situation. The amount of toy matrix that children ingest strongly depends on the behaviour of the child. Two types of behaviour can be distinguished:

- *Hand/object-to-mouth behaviour*, in which case small amounts of toy matrix that adhere to the hands or other objects are ingested continuously during hand-mouth and object-mouth contact.
- *Single ingestion*, in which case a relatively large piece of toy matrix is ingested during a single event.

In the present chapter, the bioaccessibility values of lead (Pb) from chalk and paint for four different solid-to-fluid ratios are determined and discussed. Pb was used because of practical reasons, i.e. the analysis in digestion juice was validated and toy matrices with Pb were available. Contaminated chalk had been provided by the Inspectorate for Health Protection.

In the present chapter, first the amounts of toy matrix that can be ingested by children are addressed. Subsequently, these amounts of ingested toy matrix are translated into solid-to-fluid ratios in the gastro-intestinal compartment of a child. Based on these theoretical values, solid-to-fluid ratios are chosen that are used in experiments to study the effect of the ratios on bioaccessibility. This is followed by sections on the experimental set-up, the results and discussion, and conclusions.

2.2 Solid-to-fluid ratio

2.2.1 Amounts of toy ingested

Hand/object-to-mouth behaviour. Information on the duration of hand-to-mouth behaviour is available in literature (Juberg et al., 2001; Groot et al., 1998). However, data on the amounts of toy that are ingested via hand-to-mouth behaviour by children are not available. Such data are only available for ingestion of soil. Therefore, similar to the assumptions made by Bremmer and Van Veen (2002), it is assumed that for “dry” product, i.e., products which do not immediately stick to the skin such as chalk and paint flakes, the estimate of the default amounts of ingested toy matrix is based on values from the ingestion of soil (Bremmer et al., 2002).
Several studies have been performed on the amount of soil ingested by children (Stanek et al., 1995; Davis et al., 1990; Calabrese et al., 1989; Van Wijnen et al., 1990; Stanek et al., 1998). Tracers that are naturally present in soil have been used to estimate the amount of soil ingested by determination of the tracers in the faeces. Based on these studies an average daily

soil intake of 100 mg for children and 50 mg for adults is derived for human risk assessment in the Netherlands for contaminants in soils (Swartjes, 2002). Most other countries employ similar values (Swartjes, 2002; Lijzen et al., 1999).
For use with the human exposure model ConsExpo a default value for the amount of soil or toy ingested by children is set at 300 mg per day, based on a child of 18 months (Bremmer et al., 2002). This is derived from more or less the same studies. Assuming a default time during which children are in contact with soil of 50 minutes per day, an ingestion rate of soil is calculated of 6 mg per minute (Bremmer et al., 2002).
For "wet" products, for example finger paint, a five times higher ingestion rate is estimated (30 mg/min), based on differences between soil and mud (Bremmer et al., 2002).

Single ingestion. From literature on soil it is known that children sometimes ingest large amounts of soil on a single day. It was observed by Calabrese et al. that some children ingest up to 25 to 60 g of soil during a single day (Calabrese et al., 1997), although these are exceptional high amounts. Soil ingestion of about 1 g during a single event is more common (Calabrese et al., 1997). Therefore, 1 g is assumed for ingestion of toy during a single ingestion event in the present study.

2.2.2 Translation to solid-to-fluid ratios

In the in vitro digestion model 6 ml of saliva and 12 ml of gastric juice are used in the swallow model (the swallow model is applicable in the present study because a non-food item is ingested) (Oomen et al., 2003). In the human stomach under fasting conditions the volume of gastric juice is about 50 ml, whereas the volume for children is about 9 ml (Geigy, 1969; Davenport, 1984; Kulkarni et al., 1997; Kawana et al., 2000). The volume can increase 50-fold after eating (Malagelada et al., 1976). The in vitro digestion model is developed to have physiologically based ratios of the different digestive fluids, i.e. saliva, gastric juice, duodenal juice, and bile.

Hand/object-to-mouth behaviour. Children are assumed to ingest about 100-300 mg of soil via hand-to-mouth and object-to-mouth behaviour. Assuming that this amount is ingested at once, the solid-to-fluid ratio in the stomach phase ranges between 1:90 and 1:30. However, for hand/object-to-mouth behaviour these ratios are not correct as children ingest 100-300 mg soil throughout the day. For fasting conditions the gastric juice is renewed about every 20 minutes (Hörter et al., 1997; Malagelada et al., 1976). With an ingestion rate of 6 mg/min during contact with soil (Bremmer et al., 2002), at maximum 120 mg of soil is present in the stomach. This corresponds with a solid-to-fluid ratio in the stomach phase of 1:75. When the child is not in contact with soil for 20 minutes in succession, less soil will be present in the same volume of gastric fluid, resulting in solid-to-fluid ratios of 1:>75. For example, when 100-300 mg of soil is ingested at even rate over 12 hours (assuming that the child sleeps the other 12 hours), 3-8 mg of soil is present in 9 ml of gastric fluid. The latter case would thus result in a solid-to-fluid ratio between 1:3000 and 1:1125.
Finally, it should be noticed that ingestion of 100-300 mg of soil is an upper daily average for hand/object-to-mouth behaviour. In most cases less soil will be ingested resulting in lower solid-to-fluid ratios than calculated above.

In case of fed conditions the volume of gastric content is approximately 0.5 litre. Hence, the solid-to-fluid ratio for hand/object-to-mouth behaviour would range between 1:1667 and 1:166667.

Single ingestion. During a single ingestion event about 1 g of soil is ingested. With a gastric volume for fasting conditions of 9 ml, the solid-to-fluid ratio would be 1:9. However, in practice, most of the time less matrix will be present in 9 ml gastric juice than 1 g because the juice is continuously renewed. In addition, most of the time children have at least some food constituents left in the stomach, resulting in a larger volume than 9 ml.

For single ingestion of toy matrix under fed conditions, i.e. assuming ingestion of 1 g toy matrix and a volume of gastric content of 0.5 l, the solid-to-fluid ratio would be 1:500.

Experimentally compared solid-to-fluid ratios. The widest range of solid-to-fluid ratios in the stomach covering both fasting and fed conditions is between 1:9 and 1:166667. For practical reasons solid-to-fluid ratios with less solid than 1:2250 are not used, as the concentration of Pb in digestive juice would be below the limit of quantification. Solid-to-fluid ratios with more solid than 1:45 are not used because this will only occur in very few cases, as children are seldomly completely fasted during daytime, which is also the time that they can be exposed to contaminants in soil or toy matrices. Hence, in the present study the solid-to-fluid ratio was varied covering a range for the stomach between 1:45 and 1:2250, see Table 1.
The solid-to-fluid ratios based on the volume of juice in the intestine are presented in Table 2. These values follow from the solid-to-fluid ratios for the stomach.

In summary:
Children are considered to ingest about 0.1-0.3 g of soil or toy via hand-to-mouth and object-to-mouth behaviour, whereas 1 g of soil or toy can be ingested during a single event. Based on these amounts of ingestion, the widest range of solid-to-fluid ratios in the stomach covering both fasting and fed conditions is between 1:9 and 1:166667. In the present study the solid-to-fluid ratio is varied covering a range for the stomach between 1:45 and 1:2250 (see Table 1).
Solid-to-fluid ratios of 1:45 are best representing single ingestion events, whereas higher ratios such as 1:1800 and 1:2250 are representing hand-to-mouth behaviour. Higher ratios are also obtained for fed conditions compared to fasting conditions. It should be noticed that in some cases in real life more extreme ratios, i.e. 1:<45 and 1:>2250, are possible.

2.3 Experiment

2.3.1 Experimental set-up

Matrices. In the present study paint flakes and chalk for exterior use (NL: stoepkrijt) were applied. Chalk for exterior use will be referred to as chalk for the remainder of the manuscript. These matrices were contaminated during the production process. Paint contaminated with Pb was obtained from the NIST (National Institute of Standards and

Technology, US), and referred to as SRM (Standard Reference Material) 2581. SRM 2581 is composed of paint collected from the interior surfaces of housing. Paint flakes contained 3.8 mg Pb/g as determined by destruction. Flakes size was less than 100 μm. Chalk was obtained from the Inspectorate for Health Protection without further specifications. The chalk was highly contaminated with Pb, i.e. 22 mg/g chalk, and was crushed to powder before use.

Fasting conditions - Swallow model. To simulate fasting conditions, experiments were performed according to the swallow model described by Oomen et al. (Oomen et al., 2003). The swallow model was used because chalk for exterior use and paint chips are ingested by children without first sucking on the matrix.
In short, the digestion started by introducing 6 ml saliva to 0.01-0.4 g of toy (dry weight). This mixture was rotated head-over-heels for 5 min at 55 rpm. Subsequently, 12 ml of gastric juice (pH 1.4 ± 0.02) was added, and the mixture was rotated for 1 h. The pH of the mixture of saliva and gastric juice was determined and, if necessary, set to approximately pH 1.6. The mixture was rotated for another h and the pH was determined. Finally, 12 ml of duodenal juice (pH 8.1 ± 0.2) and 6 ml bile (pH 8.0 ± 0.2) were added simultaneously, and the mixture was rotated for another 2 h. The pH of the chyme was determined once more.
All digestive juices were heated to 37 ± 2 °C at the start of the experiment. Mixing took place in a rotator that was also heated to 37 ± 2 °C. At the end of the in vitro digestion process, the digestion tubes were centrifuged for 5 min at 2750 g, yielding the chyme (the supernatant), and the digested matrix (the pellet).

Fed conditions - In vitro digestion model for food. To simulate fed conditions, experiments were performed according to the in vitro digestion model for food as described by Versantvoort et al. (Versantvoort et al., 2003).
In short, the digestion started by introducing 0.01 or 0.1 g toy matrix to 6 ml saliva and 4.5 gram infant formula. Then 12 ml of gastric juice was added, and the mixture was rotated head-over-heels for 2 hours at 55 rpm. Finally, 12 ml of duodenal juice, 6 ml bile, and 2 ml sodium bicarbonate (84.7 g/l) were added simultaneously, and the mixture was rotated for another 2 h. The pH of the chyme was determined once more.
Similar to the swallow model, the digestive juices were heated to 37 ± 2 °C at the start of the experiment, and mixing took place in a rotator that was also heated to 37 ± 2 °C. Separation of chyme and pellet was obtained by centrifugation at 2750*g* for 5 min.

Experimental design. Four different amounts of SRM paint and chalk were digested using the swallow model, that is, fasting conditions. Amounts of 0.01 g, 0.04 g, 0.1 g, and 0.4 g per digestion tube were employed. The corresponding solid-to-fluid ratios for the stomach were 1:1800, 1:450, 1:180, and 1: 45 (see Table 1). In addition, two different amounts of SRM paint and chalk were digested using the in vitro digestion model for food, that is, fed conditions. Amounts of 0.01 g and 0.1 g per digestion tube were used, corresponding to solid-to-fluid ratios for the stomach of 1:2250 and 1:225 (Table 1). By comparison of the results of the swallow model and the digestion model for food, the effect of the presence of food can be evaluated.
Each amount of toy was determined in three separate digestion tubes and on two (in vitro digestion model for food, i.e. n=6) or three (swallow model, i.e. n=9) different days. The experiments with the model were repeated on different days to obtain more reliable absolute bioaccessibility values, as previous results showed that variation was highest between days.

Table 1 and 2 schematically present the solid-to-fluid ratios in the stomach and intestine, respectively.

*Table 1. The amounts of SRM paint and chalk used per digestion tube and the corresponding solid-to-fluid ratios in the **stomach** phase.*

Matrix	Physiological state	Amounts	Solid:fluid ratio – stomach
SRM paint	Fasted	0.01, 0.04, 0.1, 0.4	1:1800; 1:450; 1:180; 1:45
Chalk	Fasted	0.01, 0.04, 0.1, 0.4	1:1800; 1:450; 1:180; 1:45
SRM paint	Fed	0.01, 0.1	1:2250; 1:225
Chalk	Fed	0.01, 0.1	1:2250; 1:225

*Table 2. The amounts of SRM paint and chalk used per digestion tube and the corresponding solid-to-fluid ratios in the **intestinal** phase.*

Matrix	Physiological state	Amounts	Solid:fluid ratio – intestine
SRM paint	Fasted	0.01, 0.04, 0.1, 0.4	1:3800; 1:950; 1:380; 1:95
Chalk	Fasted	0.01, 0.04, 0.1, 0.4	1:3800; 1:950; 1:380; 1:95
SRM paint	Fed	0.01, 0.1	1:4250; 1:425
Chalk	Fed	0.01, 0.1	1:4250; 1:425

2.3.2 Results and discussion

2.3.2.1 pH values

The pH values were determined at several moments in the in vitro digestion procedure (see also section on swallow model and in vitro digestion model for food):

- directly after adding gastric juice to the mixture of matrix and saliva
- at the end of the stomach phase, i.e. after 2 hours of digestion in the stomach
- at the end of the intestinal phase, i.e. after 2 hours of digestion in the intestine

If the pH after the first pH measurement was too high (mean pH>2), which can be caused by the matrix, the pH was adjusted to about 1.6 with concentrated HCl. Table 3 presents the range of pH values and volumes of HCl that were added for the swallow in vitro digestion model during three days. Table 4 presents the range of pH values and volumes of HCl that were added for the in vitro digestion model for food during two days.

*Table 3. Range of pH values determined at various moments during the in vitro digestion procedure of the **swallow model**.*

Matrix and amount	pH begin stomach	Volume HCl added	pH end stomach	pH end intestine
SRM paint 0.01 g	1.43-1.51	-	1.39-1.49	5.78-6.26
SRM paint 0.04 g	1.47-1.65	-	1.43-1.64	6.13-6.78
SRM paint 0.1 g	1.82-2.13	-	1.82-2.15	6.51-6.90
SRM paint 0.4 g	4.89-5.09	0.10 ml	1.82-2.80	6.02-6.64
Chalk 0.01 g	1.42-1.55	-	1.40-1.53	5.65-6.38
Chalk 0.04 g	1.50-1.64	-	1.52-1.67	5.95-6.56
Chalk 0.1 g	1.66-1.77	-	1.68-1.86	6.24-6.68
Chalk 0.4 g	2.08-2.57	0.05 ml	1.73-1.92	5.45-6.42
No matrix	1.40-1.46	-	1.38-1.42	5.87-6.42

The pH ranges comprise the values determined over three days.

*Table 4. Range of pH values determined at various moments during the **in vitro digestion procedure for food**.*

Matrix and amount	pH begin stomach	Volume HCl added	pH end stomach	pH end intestine
SRM paint 0.01 g	2.93-3.84	0.05 ml	1.77-2.24	6.53-6.59
SRM paint 0.1 g	2.00-2.89	0.05 ml	1.40-1.76	6.51-6.56
Chalk 0.01 g	2.16-2.86	0.05 ml	1.57-1.91	6.54-6.58
Chalk 0.1 g	2.00-2.74	0.05 ml	1.43-1.70	6.51-6.57
No matrix	2.10-2.65	0.05 ml	1.46-1.69	6.49-6.55

The pH ranges comprise the values determined over two days.

For the swallow model, the gastric pH increased with increasing amounts of chalk or SRM paint (Table 3). It was necessary to add 0.10 and 0.05 ml of concentrated HCl to the digestion tubes with 0.4 g of SRM paint and chalk, respectively. The pH at the end of the stomach phase varied between 1.39 and 2.80, and at the end of the intestinal phase between 5.45 and 6.90. Although the variation is still considerable, it is acceptable considering the large differences in amounts of matrix added (factor 40), and the effect the matrix can have on the pH (for example, 0.4 g of SRM paint increased the pH in the stomach from about 1.5 to about 5.0).

In contrast, the pH for the in vitro digestion model for food seemed to be less dependent on the amount of matrix added (Table 4). It was remarkable that the gastric pH was highest for the low amount of SRM paint. Especially the pH at the end of the intestine was very constant (range between 6.49 and 6.59), even though the pH at the end of the stomach phase varied between 1.40 and 2.24.

2.3.2.2 Relationship solid-to-fluid ratio and bioaccessibility

Table 5 presents the bioaccessibility data of Pb from SRM paint and chalk using the swallow model with different amounts of SRM paint or chalk per digestion tube. These data are presented graphically in Figure 1.

*Table 5. Gastric and intestinal bioaccessibility (± SD) values determined after in vitro digestion according to the **swallow model** on three different days, using different amounts of SRM paint or chalk per digestion tube.*

Matrix and amount	N	Gastric Bioaccessibility (%)			Intestinal Bioaccessibility (%)		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
SRM paint 0.01 g	3	n.a.*	99 ± 20	92 ± 23	40 ± 7	33 ± 6	25 ± 6
SRM paint 0.04 g	3	n.a.*	79 ± 9	75 ± 3	46 ± 4	46 ± 4	47 ± 2
SRM paint 0.1 g	3	n.a.*	68 ± 2	75 ± 5	45 ± 5	52 ± 3	48 ± 3
SRM paint 0.4 g	3	n.a.*	43 ± 1	49 ± 2	23 ± 3	31 ± 2	24 ± 2
Chalk 0.01 g	3	n.a.*	64 ± 22	69 ± 15	53 ± 8	41 ± 11	40 ± 8
Chalk 0.04 g	3	n.a.*	72 ± 1	87 ± 5	24 ± 3	25 ± 2	22 ± 4
Chalk 0.1 g	3	n.a.*	75 ± 3	79 ± 2	12 ± 1	11 ± 2	11 ± 1
Chalk 0.4 g	3	n.a.*	54 ± 14	59 ± 2	3.7 ± 1.2	4.4 ± 0.5	4.3 ± 0.3
No matrix	1	n.a.*	0	0	0	0	0

* Data not available.

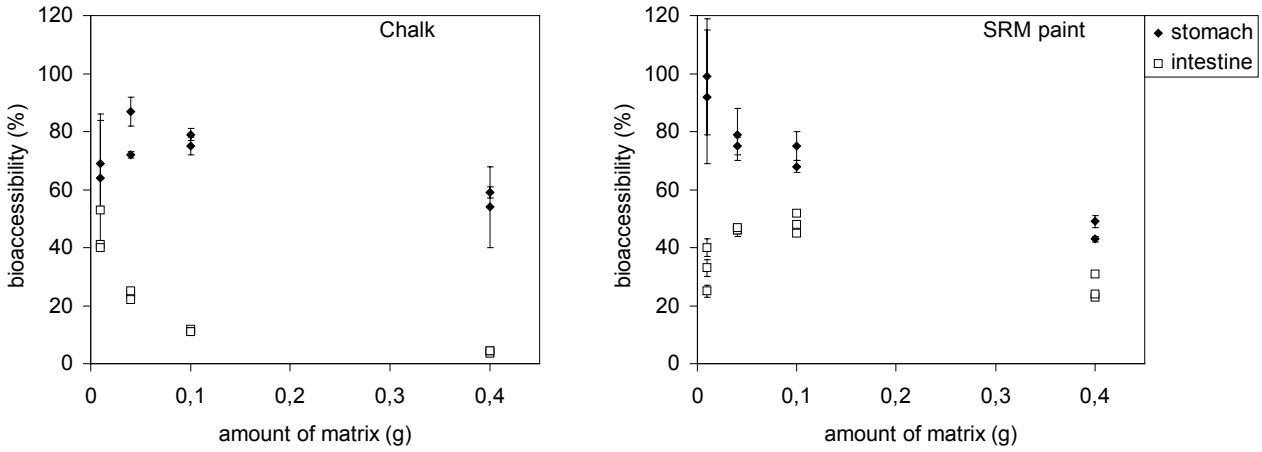

Figure 1. The effect of the amount of matrix per digestion tube on the bioaccessibility of Pb in the stomach and intestinal compartment, using the swallow model.
Each data point represents the average of three digestion tubes of a single day and its standard deviation is shown as well. Different data points for the same amount of matrix are data obtained on different experimental days.

The bioaccessibility of Pb from both matrices was high (>70%) in the gastric compartment. Only at the highest amount of SRM paint and chalk, the bioaccessibility decreased to 43% - 59%. Thus, most of Pb was released from its matrix under the acid conditions in the stomach and only at the highest solid-to-fluid ratio (1:45) the bioaccessibility of Pb decreased.

Table 5 and Figure 1 show that the bioaccessibility of Pb in the intestinal compartment was always lower than the corresponding bioaccessibility in the gastric compartment. However, no correlation between the bioaccessibilities in the gastric and intestinal compartment was apparent.

As can be seen in Table 5 and Figure 1, bioaccessibility of Pb was strongly dependent on the amount of matrix per digestion tube. This is exemplified by chalk that showed an intestinal

bioaccessibility of 40-50% after digestion with 0.01 g, which gradually decreased to 4% after digestion with 0.4 g of chalk. However, no clear correlation between amount of matrix digested and Pb bioaccessibility was apparent for SRM paint: the bioaccessibility seemed to show an optimum at 0.04 and 0.1 g SRM paint compared to the bioaccessibilities at 0.01 and 0.4 g SRM paint. No clear correlation between bioaccessibility and pH was observed.

As the Pb concentration in chalk was more than 5 times higher than the concentration of Pb in SRM paint, saturation of Pb in chyme might contribute to the decrease in bioaccessibility of Pb at higher amounts of chalk digested. Figure 2 shows the concentration of Pb in the chyme as function of the amount of Pb digested. For SRM paint, the concentration of Pb in the chyme was dose-proportional to the amount Pb digested only at the highest amount the concentration of Pb was less than dose-proportional. For chalk, the concentration of Pb in the chyme was much less than dose-proportional, which might indicate saturation of Pb in chyme. However, the absolute concentration of Pb in chyme was higher for SRM paint (highest concentration in chyme 11 mg/l) than for chalk (highest concentration in chyme 10 mg/l). However, due to components in the chalk, saturation may occur at lower Pb concentration than was found for Pb from paint. Thus, the matrix is probably an important factor for bioaccessibility of contaminants.

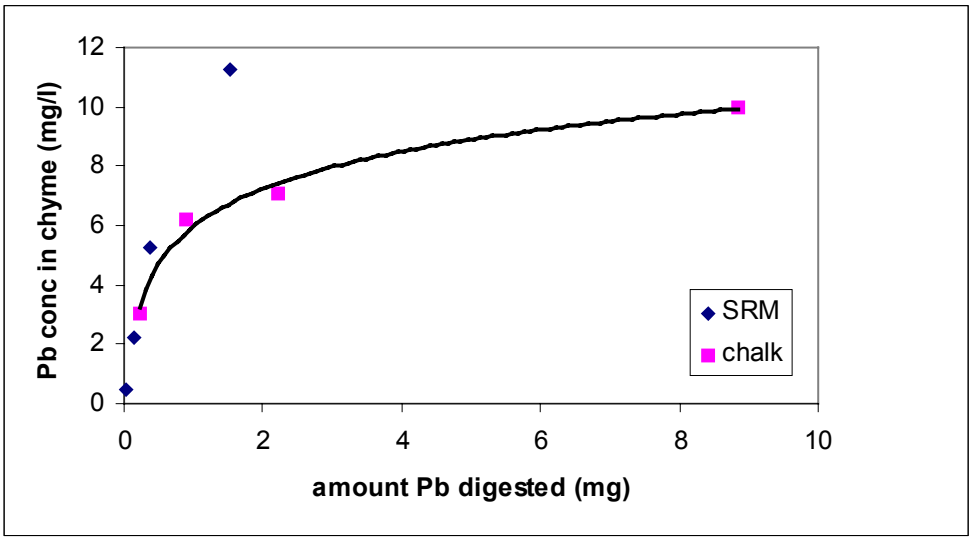

Figure 2. Concentration of Pb in the artificial chyme of the intestinal compartment in relation to the amount Pb digested using the swallow model.

Based on these different results with Pb from chalk and paint, it is concluded that it cannot be predicted beforehand how bioaccessibility depends on the solid-to-fluid ratio. Thus, the solid-to-fluid ratio should be considered when performing in vitro digestion experiments, and when interpreting the results of the in vitro digestion model.
A solid-to-fluid ratio of 1:45 (0.4 g matrix per digestion tube) was chosen as representative for a single ingestion event, whereas a ratio of 1:1800 closer represents hand-to-mouth behaviour. These ratios are based on ingestion rates by children, and limited by physiological conditions in the gastro-intestinal tract. As the exposure scenario affects the solid-to-fluid ratio and the solid-to-fluid ratio affects the bioaccessibility, it should be considered which exposure scenario is relevant or a range of solid-to-fluid ratios should be tested so that a

worst case or a weighted bioaccessibility value can be determined. A range of solid-to-fluid ratios similar to the range employed in the present study is recommended.

2.3.2.3 Effect of food on bioaccessibility

By applying the in vitro digestion model for food, the bioaccessibility under fasting conditions can be compared with the bioaccessibility under fed conditions. In this manner insight is obtained into the possible effects of food on bioaccessibility of Pb from paint flakes and chalk. The gastric and intestinal bioaccessibility data for the in vitro digestion model for food are presented in Table 6.

*Table 6. Gastric and intestinal bioaccessibility (± SD) values determined after in vitro digestion according to the **in vitro digestion model for food** on two different days, using different amounts of SRM paint and chalk per digestion tube.*

Matrix and amount	N	Gastric Bioaccessibility (%)		Intestinal Bioaccessibility (%)	
		Day 1	Day 2	Day 1	Day 2
SRM paint 0.01 g	3	72 ± 15	75 ± 9	34 ± 3	37 ± 7
SRM paint 0.1 g	3	104 ± 23	107 ± 7	43 ± 1	40 ± 3
Chalk 0.01 g	3	100 ± 12	86 ± 12	39 ± 5	46 ± 1
Chalk 0.1 g	3	57 ± 20	91 ± 3	17 ± 2	20 ± 3
No matrix	1	0	0	0	0

Intestinal bioaccessibility was similar for the model for fasting conditions (swallow model) and the in vitro digestion model for food, except for chalk 0.1 g in which case the bioaccessibility was higher under fed (17-20%) than for fasting (11-12%) conditions. A higher bioaccessibility can be explained by the higher complexing capacity of Pb for stimulated chyme with food constituents. Similar to the result of the swallow model, the results in Table 6 suggest that bioaccessibility as determined with the in vitro digestion model for food can also depend on the amount of matrix per digestion tube. For the in vitro digestion model with food, bioaccessibility was only determined for two different amounts of matrix. Therefore, the relationship between amount of matrix and bioaccessibility is not very clear. Nevertheless, bioaccessibility is not always similar and does seem to show the same trend with increasing solid-to-fluid ratio as the results obtained with the swallow model.

2.3.2.4 Comparison to previous studies

The bioaccessibility data of the swallow model for 0.4 g SRM paint and 0.4 g chalk for exterior use can be compared to previous data of the swallow model obtained during the development of the in vitro digestion models for toys (Oomen et al., 2003). These data are presented in Table 7.

Table 7. Comparison between present and previous data (Oomen et al., 2003) of SRM paint and chalk for exterior use after digestion with the swallow model.

Matrix and amount	Phase	Bioaccessibility previous study (%)	Bioaccessibility present study (%)		
			Day 1	Day 2	Day 3
SRM paint 0.4 g	Gastric	52 ± 1 (n=6)	n.a.*	43 ± 1	49 ± 2
Chalk 0.4 g	Gastric	59 ± 8	n.a.*	54 ± 14	59 ± 2
SRM paint 0.4 g	Intestinal	25 ± 4 (n=6)	23 ± 3	31 ± 2	24 ± 2
Chalk 0.4 g	Intestinal	3.3 ± 0.4	3.7 ± 1.2	4.4 ± 0.5	4.3 ± 0.3

N=3, except where indicated differently.
* Data not available.

As can be seen, the bioaccessibility values are in good agreement, indicating that the in vitro digestion procedure is well reproducible.

2.4 Conclusions

In summary:

- The amount of matrix can have substantial effect on the bioaccessibility. For example, the bioaccessibility of chalk in the intestinal compartment decreased from about 50% to 4% when increasing the amount of matrix from 0.01 to 0.4 g per digestion tube. However, for SRM paint the effect of amount of matrix on the bioaccessibility of Pb was much less. The concentration of Pb in chyme might get saturated at high Pb levels, in a matrix dependent manner. It was concluded that the solid-to-fluid ratio is a variable that can lead to different bioaccessibility values and should be considered for accurate exposure assessment.
- As the solid-to-fluid ratio can affect bioaccessibility, it is recommended to test a range of solid-to-fluid ratios for any contaminant/matrix combination so that a worst case or weighted bioaccessibility value can be determined. A range of solid-to-fluid ratios similar to the range used in the present study, i.e. between 1:45 and 1:2250, is recommended as this range is derived from ingestion rates by children and limited by physiological conditions in the gastro-intestinal tract.
- Day-to-day variation was low for bioaccessibility obtained by the swallow model, i.e. fasting conditions. Data obtained in this study are in agreement with data obtained in previous studies (Oomen et al., 2003). The bioaccessibility of Pb in the intestinal compartment was always lower than the corresponding bioaccessibility in the gastric compartment. However, no (other) correlation between the bioaccessibilities in intestinal and gastric compartment was found.
- In the present study intestinal bioaccessibility of Pb from chalk or paint flakes ranged between 4 and 53%, including all solid-to-fluid ratios. The highest bioaccessibility for SRM paint was 52% and for chalk 53%. This suggests that a considerable fraction, at least 47-48%, of the Pb in chalk and paint does not contribute to the internal exposure when a child ingests these contaminated matrices.
- Intestinal bioaccessibility values for both SRM-paint and chalk were not very different for fasting and fed conditions, except for chalk 0.1 g in which case bioaccessibility was about a factor 2 higher under fed conditions.

3. Phthalate release from soft PVC baby toys

3.1 Introduction

Phthalates are commonly used as plasticizers for soft PVC to impart flexibility and durability. Exposure of laboratory animals to phthalates show toxicity in liver, kidney and testicles and phthalates may be animal carcinogens causing fetal death, malformations, and reproductive toxicity. Soft PVC is often used in food packaging, clothing, medical devices, toys and childcare articles. Phthalates can migrate out of the product and in this way humans can be exposed. As young children have a prolonged contact with their toys and child-care products by mouthing, young children might be higher exposed to phthalates than adults. The European Commission considered to reduce the risks of phthalates to children either by prohibition of the use of certain phthalates in toys and child-care articles intended to be put in the mouth by children under the age of three or by establishments of limits for migration of phthalates from toys. An approach based on migration limits requires reliable testing methods. As validated methods for migration of phthalates from toys were not available at the time, the European Commission decided in 1999 to adopt an interim prohibition on six phthalates (see Table 8) in toys and child-care articles that are intended to be put in the mouth by children under three years of age (Decision 1999/815/EC). The validity of this decision is frequently renewed, the last time in February 2004 (2004/178/EC).

Table 8. Risk assessment on phthalates in toys and child-care articles.

substance	abbreviation	CAS-number	RAR status*
di(2-ethylhexyl) phthalate	DEHP	117-81-7	Draft 2003
di-iso-nonyl phthalate	DINP	68515-48-0 and 28553-12-0	Final 2003/08/07
di-iso-decyl phthalate	DIDP	68515-49-1 and 26761-40-0	Final 2003/08/07
dibutyl phthalate	DBP	84-74-2	Final 2004/02/11
butylbenzyl phthalate	BBP	85-68-7	Draft 2003
n-dioctyl phthalate	DNOP	117-84-0	#

*Risk assessment reports (RARs) can be obtained at http://ecb.jrc.it/exsisting_chemicals
The most abundant n-dioctyl phthalate was DEHP. No risk assessment of other n-dioctyl phthalate is under consideration.

In Table 8 the six phthalates and their abbreviation and current status of the European risk assessment reports (RAR) is shown. Although the ban includes the use of these six phthalates in toys and child-care articles, the concerns for health risk are not the same for all six phthalates. Children are considered to be at risk from exposure to DEHP from toys and child-care articles and, therefore, DEHP in toys and child-care articles has to be and is reduced. The phthalate DINP is the major replacement product of DEHP in PVC toys and child-care articles. Therefore, exposure of children to DINP is mainly related to exposure from toys. Risk assessment in the US by the CHAP-CPSC concluded that there may be a DINP risk for young children who routinely mouth DINP containing toys for 75 minutes per day or more. However, based on a new observation study and migration data, the CHAP-CPSC concluded in 2002 (http:www.cpsc.gov/library) along with the RAR of the European Commission that

exposure to DINP from DINP-containing toys would be expected to pose a minimal to non-existent risk for injury for the majority of children at normal behaviour. For DIDP the amounts of DIDP in toys are low and therefore there is no concern. However, in case DIDP should be used as a substitute for other phthalates in toys, there are concerns for hepatic toxicity. In the RARs on DBP and BBP it was concluded that the exposure to DBP and BBP from toys is low and a high margin of safety was calculated. Thus, for several phthalates the risk of exposure of children is considered to be low at normal behaviour and at the considered amounts of phthalates in toys. Providing that reliable migration methods are available, these phthalates may be considered for setting a limit of migration of the phthalates from toys.

In meanwhile also much attention has been paid to development and validation of methodologies to measure migration of DINP from toys and childcare articles (Könemann, 1998; Simoneau et al., 2001). In 1998, the release of DINP from soft PVC baby toys was investigated by a working group of representatives from interested parties (inspectorate, consumer groups, industry, retailers) and reported by RIVM (Könemann, 1998). A method to determine the release rate of DINP from soft PVC baby toys into saliva simulant was developed by TNO. This laboratory method was compared with the release of DINP from soft PVC toys into saliva of human volunteers. Based on this study, the Joint Research Centre (JRC) co-ordinated validation of the method by an intra- and inter-laboratory comparison participated by 12 EU laboratories and 4 US labs (Simoneau et al., 2001). A head-over-heels rotation method was proposed as a routine laboratory method because the interlaboratory reproducibility of the method was better than the method based on horizontal shaking and the DINP release in saliva simulant was close to the in vivo results (Könemann, 1998; Simoneau et al., 2001).

In this project an *in vitro* digestion model has been developed to investigate mobilisation of contaminants from food, soil and toys (Sips et al., 2001; Oomen et al., 2003; Versantvoort et al., 2003). This model concerns a three-step procedure simulating the digestion process in successively the mouth, stomach and intestine and accounts for the entire digestion process.

The aim of the present study is to compare the first step of the *in vitro* digestion model, i.e. simulation of the digestion process in the mouth (Oomen et al., 2003) with data from the in vivo study (Könemann, 1998; Simoneau et al., 2001). The Inspectorate for Health Protection provided us with the same DINP containing PVC disks as have been used in these previous studies (Könemann, 1998; Simoneau et al., 2001). Therefore, the release of DINP from the PVC disks in saliva simulant with the *in vitro* digestion model will be compared with the results obtained *in vivo* as a validation of the *in vitro* digestion model. Furthermore, the results will be compared with the results of the interlaboratoy in vitro validation study by the JRC.

3.2 Testing procedures

3.2.1 Test specimen

The same batch of PVC disks was used in this study as have been used in the study in 1998 (Könemann, 1998; Simoneau et al., 2001). A batch of 3 mm thick PVC pads was prepared in a laboratory using a well defined protocol. The final sheet was composed of PVC (58.8%),

di-isononylphthalate (Jayflex® DINP) (38.2%), epoxidized soybean oil (1.8%) and Ca/Zn stabiliser (1.2%). Disks were punched from the sheet with a diameter of 23 mm. The total area of a disk was approximately 10 cm^2. Since the disks were used after 5 years of storage at room temperature in the dark, first the total content of DINP was determined. Mean concentrations of DINP of three disks determined in duplicate was 40.3 ± 1.9%, which is in agreement with the reported initial concentration of 38.2% (Könemann, 1998).

3.2.2 Saliva simulant

Saliva production increases by mouthing toys. The composition of the saliva is dependent on the flow rate: at higher flow rates, sodium, calcium, chloride, bicarbonate, (and amylase) increase whilst phosphate concentrations and mucin decrease and the potassium concentrations show little further change. Table 9 shows that there are differences in composition of saliva simulant prepared according to the JRC and RIVM. Ionic strength of the saliva is higher for the saliva simulant of RIVM, especially the concentrations of sodium and carbonate are higher. Furthermore, urea and the enzyme amylase are present in the saliva of RIVM and not in saliva of JRC. JRC used two sets of saliva simulant, one with and one without mucin. The concentration of mucin in the digestion model by RIVM (0.025 g/l) is much lower than 1.6 g/l mucin added by JRC.

Table 9. Composition of saliva simulant used in the in vitro digestion model of the RIVM and used in the study of 1998 and by the Joint Research Centre (JRC).

Concentration of constituents	Saliva digestion model RIVM	Saliva JRC	Saliva JRC + mucin
Potassium	14.1 mM	24.2 mM	24.2 mM
Sodium	37.5 mM	5.6 mM	5.6 mM
Magnesium	--	0.8 mM	0.8 mM
Calcium	--	1.0 mM	1.0 mM
Chloride	17.1 mM	19.1 mM	19.1 mM
Phosphate	7.4 mM	3.3 mM	3.3 mM
Carbonate	20.2 mM	3.8 mM	3.8 mM
Thiocyanate	2.1 mM	--	--
Mucin	0.025 g/l	--	1.6 g/l
Amylase	0.29 g/l	--	--
Uric acid	0.015 g/l	--	--
Urea	0.2 g/l	--	--

3.2.3 Digestion procedure

The results with saliva simulant of RIVM can be compared to in vivo results and in vitro results with JRC saliva simulant. Hence, the in vitro digestion model (suck model) was restricted to the saliva phase rather than the entire gastro-intestinal system.
In short, the digestion started by introducing 18 ml of stimulated saliva (pH 6.8) to one PVC disk in 100 ml glass flasks. This mixture was rotated head-over-heels for 15 min (unless indicated else) at 55 rpm. Subsequently, the saliva was centrifuged during 5 min at 2750 g, and sampled. Further details about the digestion procedure are provided by Oomen et al.

(Oomen et al., 2003). Saliva was preheated to 37 ± 2°C, and incubation also occurred at this temperature.

3.2.4 Analysis of DINP in saliva simulant

Glass flasks were used for the in vitro digestion because after sub-sampling the saliva simulant from polycarbonate tubes, the recovery of DINP was <70% whereas by total volume extraction in the 100 ml glass flasks a recovery of >95% was found.
Analysis of DINP in saliva simulant was according to Schakel (Schakel, 2000). Total volume (18 ml) 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 0.25 to 5 µg DINP/ml iso-octane. Recovery of DINP from saliva simulant was higher than 95%, however, when water was used as simulant the recovery was only 10%. By addition of 2 ml of 1.5 M NaCl to the water just before extraction with iso-octane, the recovery of DINP was again >95%.

3.3 Results and discussion

3.3.1 Comparison of in vitro digestion model RIVM with human volunteer study

In the first experiment an experimental set-up was chosen similar to the protocol in human volunteers: every 15 min the PVC disk was removed from 18 ml saliva simulant, rinsed shortly with water and placed in another glass bottle containing 18 ml saliva simulant. This was repeated 4 times. The results are presented in Figure 3.

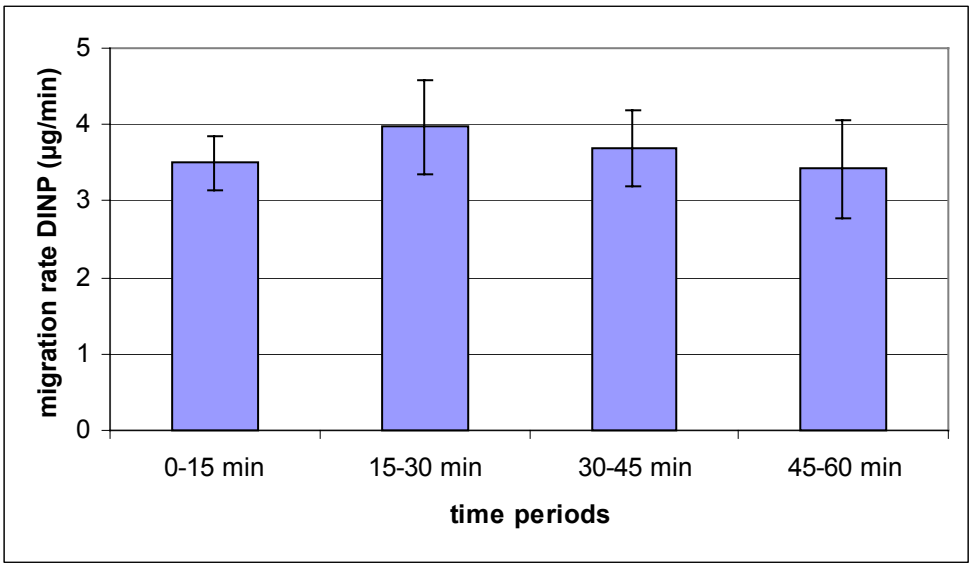

Figure 3. Migration rate of DINP from PVC disks. Data are mean ± SD of 6 disks.

Figure 3 shows that migration rates of DINP from the disks were similar in every time period. Even when the disks were used again after three weeks, the migration rate of DINP was constant showing that repeated use of the disks has no influence on the release of DINP. This suggests that even after frequent mouthing of a toy by children, DINP can still migrate out of the toy. Release of DINP in time was consistent as shown by the low variation of 12%. The inter-disk variation was somewhat higher, 22%, but acceptable.

The migration of DINP from PVC disks is low, < 0.03% was migrated from the PVC disks in 1 hour (Table 10). Mean leaching rate of DINP from PVC disks was 0.3 $\mu g \times cm^{-2} \times min^{-1}$. In the exposure model ConsExpo a leaching rate of DINP from PVC material of 0.244 $\mu g \times cm^{-2} \times min^{-1}$ is assumed (Bremmer et al., 2002). This value was found for another PVC object than the presently used disks in the volunteer study described by Könemann et al. (Könemann, 1998) and Simoneau et al. (Simoneau et al., 2001). Of the three different objects tested in the volunteer study, the highest mean leach rate was adopted by ConsExpo. The leaching rate found in the present experiment is comparable to the leaching rate used in ConsExpo. However, the CSTEE recommended to use a guidance value of 0.67 $\mu g \times cm^{-2} \times min^{-1}$ in order to protect the individual with the highest exposure. This value is, however, higher than the results obtained with the in vitro digestion model of RIVM and also higher than the results of the validation of the head-over-heals method by JRC (Simoneau et al., 2001).

The mean migration rate of DINP from the PVC disks over all time periods was 3.6 ± 0.5 µg/min. This value is comparable to the migration rate of 4.0 µg/min measured in the inter-laboratory comparison of 12 labs with the head-over-heels method using the JRC saliva without mucin, and higher but in the same order of magnitude as the average migration rate of 1.4 µg/min measured in the saliva of human volunteers (Könemann, 1998; Simoneau et al., 2001). In this study with human volunteers the range of migration rates was 0.3-8.3 µg/min. This indicates that the digestion model of RIVM is a good representative for the average situation, whereas higher values may be obtained in vivo in some cases.

3.3.2 Comparison with head-over-heels method

The *in vitro* digestion model by RIVM is also a head-over-heels rotation method but differs at some technical details such as flask size, rotation speed, saliva volume and composition to the in vitro method of the JRC (Könemann, 1998; Simoneau et al., 2001). To enable a direct comparison between the head-over-heels method (Simoneau et al., 2001) used by the JRC with the first step of the *in vitro* digestion model of RIVM, the release of DINP from the PVC disks was compared in saliva simulant compositions of both institutes. Composition of saliva simulants are shown in Table 9. Water was also included as saliva simulant because the Methods of Analysis Group (task group 2 of working group 9) concluded that distilled water was a good alternative to use as a simulant based on the results of several compounds from plastic films [CEN-report CEN/TC 52/WG 9, TG2]. The results of the mobilisation of DINP from the disks by several extractants are presented in Figure 4 and Table 10.

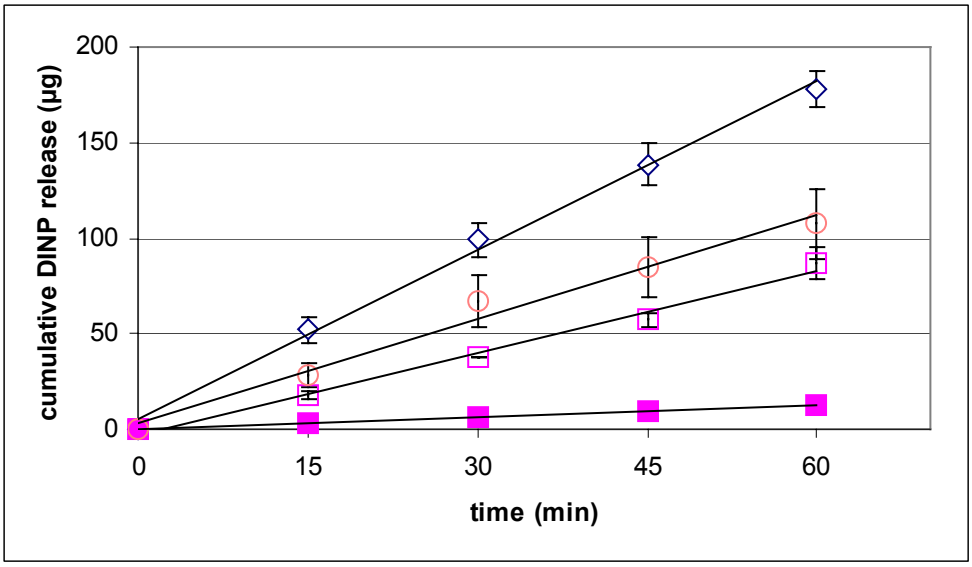

Figure 4. Effect of saliva simulant on the release of DINP from PVC disks.
The saliva simulant was replenished every 15 min up to 1 hour. The cumulative release of DINP was calculated per disk. Data are mean ± SD of 3 disks. Saliva simulant RIVM (diamonds), water (circles), JRC saliva simulant without mucin (open squares), and JRC saliva simulant with mucin (closed squares).

Table 10. Effect of saliva simulant on the migration rate of DINP from the PVC disks.

Saliva simulant	DINP migration rate (µg/min)	Release of DINP after 60 min (µg)	Bioaccessibility of DINP after 60 min (%)
RIVM	3.0 ± 0.5	178 ± 10	0.031 ± 0.002
JRC – mucin	1.4 ± 0.4	87 ± 8	0.015 ± 0.001
JRC + mucin	0.2 ± 0.1	13 ± 2	0.002 ± 0.000
Water	1.8 ± 0.6	108 ± 18	0.019 ± 0.003
RIVM + marbles	2.2 ± 0.4	131 ± 9	0.023 ± 0.001

The saliva simulant was replenished every 15 min up to 1 hour. The migration rate was calculated over each 15 min interval. Cumulative release and bioaccessibility of DINP was calculated per disk. Data are mean ± SD of 3 disks.

The release of DINP was linear with incubation time as is shown in Figure 4 for the cumulative release of DINP. In another experiment, the release of DINP was linear with time at least up to 4 hours (last time point tested). Such a time dependent release is expected for a diffusion-limited process where the compound (DINP) that is more located inside the product (PVC disk) first has to migrate to the surface area of the product before it can be released. Furthermore, the migration rate of DINP was independent of the saliva volume (9 ml, 18 ml and 36 ml were used), which is consistent with a diffusion-limited process.

Figure 4 shows that migration of DINP was affected by the composition of saliva simulant. Migration of DINP from the PVC disks was highest for the saliva simulant of RIVM and lowest for the JRC saliva simulant in presence of mucin (Table 10). The lower release of DINP in presence of mucin was also observed previously (Könemann, 1998). The migration rate of DINP from the PVC disks was 2 times higher in saliva simulant of RIVM compared to saliva simulant of JRC without mucin (Table 10). The major difference in saliva composition is the presence of amylase, urea and mucin (0.025 g/l) and the higher ionic strength of the RIVM saliva simulant (Table 9). Apparently, the low concentration of mucin in RIVM saliva

simulant (0.025 g/l) has no negative effect on the migration of DINP. The amount of amylase (0.3 g/l) is in the range of the amount of protein (0.5 g/l) found in saliva of human volunteers (Könemann, 1998). The presence of amylase, however, had no effect on the migration of DINP (checked in another experiment). Therefore, the higher ionic strength or urea seems to affect the migration of DINP in saliva simulant.

In the report of CEN/TC 52/WG 9 it was concluded that water was an excellent simulant for migration extractions of several compounds from plastic films, because the extraction of chemicals was higher from water than from the artificial saliva simulants and water is an easier matrix for analysis. The present data indicate that the release of DINP in water was comparable to the release of DINP in saliva of JRC without mucin. However, the release of DINP in saliva simulant of RIVM showed a higher extraction (3.0 ± 0.5 μg/min) than for water (1.8 ± 0.6 μg/min) and JRC saliva simulant without mucin (1.4 ± 0.4 μg/min).

Glass marbles were added for a more intensive agitation. The release of DINP from the disks was, however, somewhat lower in presence of glass marbles (Table 10) probably due to some adsorption of DINP to the marbles.

3.4 Conclusions

In summary:

The following conclusions have been drawn from the results on migration release of DINP from the PVC disks in saliva simulant with the *in vitro* digestion model:

- Migration of DINP ($\mu g \times min^{-1}$) from PVC disks was linear in time and independent of the saliva volume used.
- Migration of DINP from PVC disks in saliva was dependent on the composition of the saliva simulant. Presence of mucin (1.6 g/l) in saliva simulant according to the methodology of the Joint Research Centre (JRC) greatly reduced the migration of DINP (from 1.4 ± 0.4 μg/min to 0.2 ± 0.1 μg/min). Migration of DINP from the PVC disks was ~2-fold higher for the saliva simulant of RIVM (3.0 ± 0.5 μg/min) compared to the saliva simulant of JRC without mucin (1.4 ± 0.4 μg/min) or compared to water (1.8 ± 0.6 μg/min).
- DINP migration from PVC disks in saliva simulant with the *in vitro* digestion model of RIVM (3.3 ± 0.5 μg/min) was comparable with the head-over-heels method used in the in vivo validation study (3 ± 1 μg/min) and the inter-laboratory comparison (4.0 μg/min). This indicates that inter-laboratory variation is low, and that experimental settings did not have a large impact on the outcome.
- DINP migration rate obtained with the digestion model of RIVM (3.3 ± 0.5 μg/min) was in the same order of magnitude as the average of DINP release in saliva of human volunteers (1.4 μg/min). It should be noted that the RIVM method gives a slightly higher value than the average release rate for human volunteers, but does not cover the range of release rates found in the volunteer study (0.3-8.3 μg/min). Therefore, the *in vitro* digestion model seems to be a suitable tool to estimate the *average* exposure of children to potentially harmful substances by mouthing their toys and childcare articles.

4. Azo dyes

4.1 Introduction

Azo dyes are used in the textile, printing, paper manufacturing, pharmaceutical, and food industries and also in research laboratories. They are compounds containing at least one of the N=N azo chromophore groups. This binding is responsible for the colouring characteristics. When these compounds either inadvertently or by design enter the body through ingestion, they are metabolised to aromatic amines by intestinal micro-organisms (Chung et al., 1992; Rafii et al., 1995). Reductive enzymes in the liver can also catalyse the reductive cleavage of the azo linkage to produce aromatic amines. These aromatic amines are proven or suspected to be carcinogenic.
In July 1994, the Government in Germany banned all consumer goods which contained azo dyes by making an amendment to the German Consumer Goods Act. In June 1998, the Dutch Government followed this initiative by making a "Warenwetbesluit Azo-kleurstoffen". A recent European Union Directive (76/769/EEC) has identified 22 Carcinogenic Amines. Consumer goods which contain azo dyes that could, through cleavage of one or more azo group(s) form any of the specified 22 aromatic amines, are forbidden.
Table 11 lists the aromatic amines that are prohibited by Dutch law at concentrations higher than 30 mg/kg textile or leather. The list only gives a summary of 20 amines. Two amines, i.e. p-amino-azo-benzeen and o-anisidine, are accepted as carcinogenic compounds by the EU Directive but not by the Dutch law. A cancer risk assessment of products containing azo dyes and aromatic amines was performed by Zeilmaker et al. (Zeilmaker et al., 2000; Zeilmaker et al., 1999). These studies use assumptions on the migration of azo dyes out of the product into the human body. It was concluded that a method to assess the amount of azo dye that can migrate out of the product under physiological conditions would improve exposure and, hence, risk assessment (Zeilmaker et al., 2000; Zeilmaker et al., 1999).

The main routes of human exposure to azo dyes are:
- Oral ingestion, mainly referring to the sucking of textiles by babies and young children.
- Dermal absorption, the route of primary concern for consumers wearing azo compound-dyed products such as garment or foot wear, as well as for workers in dye production and use plants.
- Inhalation, a route of concern for workers in dye production and use industries as well as those handling newly dyed products.

Contact with aromatic amines entering the environment through the whole life-cycle of azo dyes in coloured clothes is an additional potential source of human exposure.

In the present study we focus on oral exposure of babies and young children to aromatic amines when they suck on textiles such as textile toys or clothing. The release of aromatic amines/azo dye from textile during sucking on the textile is simulated by incubation of pieces textile in saliva simulant. The fraction of aromatic amine/azo dye that is released in saliva simulant is referred to as the bioaccessible fraction. Only released compounds can be ingested and can contribute to the internal exposure and toxicity.
The aromatic amines that are presently considered are aniline, 2,4-toluenediamine, and

o-dianisidine. The choice of these aromatic amines was because textiles containing azo dyes, releasing these amines after reduction, were available at the Inspectorate for Health Protection (NL: Keuringsdienst van Waren).

A cancer risk of aromatic amines was made by Zeilmaker et al. by elaborating on several case studies (Zeilmaker et al., 2000; Zeilmaker et al., 1999). In one of these case studies the cancer risk was assessed that was associated with oral exposure to o-dianisidine (337 μg/g) from a string of a children's sweater. In another case study the cancer risk was assessed that was associated with oral exposure to 2,4-toluenediamine (359 μg/g) in textile toys. In both cases there was considerable uncertainty in the assumed exposure to the azo dyes (Zeilmaker et al., 2000; Zeilmaker et al., 1999). The release of o-dianisidine and 2,4-toluenediamine from textile into saliva simulant in the present study will be compared to the assumptions made in the risk assessment by Zeilmaker et al. and discussed. In addition, for each aromatic amine, the application of the bioaccessibility results in the exposure model ConsExpo will be discussed (Van Veen, 2001; Bremmer et al., 2002). ConsExpo is a software tool that implements a set of predictive models to assess human exposure to chemicals in consumer products.

Table 11. Overview of the list of carcinogenic amines that are prohibited according to the Dutch law ("Warenwetbesluit").

Number	Aromatic amine	CAS number
1	o-amino-azotoluene	97-56-3
2	4-aminodiphenyl	92-67-1
3	2-amino-4-nitrotoluene*	99-55-8
4	Benzidine	92-87-5
5	p-chloroaniline	106-47-8
6	4-chloro-o-toluidine	95-69-2
7	p-cresidine	120-71-8
8	2,4-diaminoanisol	615-05-4
9	4,4'-diaminodephenylmethane	101-77-9
10	3,3'-dichlorobenzidine	91-94-1
11	3,3'-dimethoxybenzidine	119-90-4
12	3,3'-dimethoxybenzidine (= **o-dianisidine**)	119-93-7
13	3,3'-dimethyl-4,4'-diaminodiphenylmethane	838-88-0
14	4,4'-methylene-bis-(2-chloroaniline)	101-14-4
15	2-naphtylamine	91-59-8
16	4,4'-oxydianiline	101-80-4
17	4,4'-thiodianiline	139-65-1
18	**2,4-toluenediamine**	95-80-7
19	o-toluidine	95-53-4
20	2,4,5-trimethylaniline	137-17-7

* 2-Amino-4-nitrotoluene is indirectly determined via measurement of 2,4-toluenediamine.

Aniline is an aromatic amine that is not included in EU Directive or the Dutch law.

4.2 Materials and Methods

4.2.1 Textiles

Three textiles were obtained from the Inspectorate for Health Protection (NL: Keuringsdienst van Waren) that contained azo dyes. The concentration of azo dyes was characterised by the amount of amines that were determined after reduction of the dyes per kg textile. One textile was known to contain aniline, another textile 2,4-diaminotoluene, and the third textile contained o-dianisidine. Table 12 gives an overview of the concentration of amines that were found per kg textile after reduction of the azo dyes.

Table 12. Overview of the concentrations of aromatic amine that were determined after reduction of azo dyes in three different textiles.

Colour	Amine	Concentration (mg/kg)
Red	Aniline	1510 (n=1)
Red	2,4-toluenediamine	499 (n=1)
Blue	o-dianisidine	650-768 (n=2)

N represents the number of replicates analysed.

4.2.2 Digestion procedure

Only sucking on textile was simulated for the experiments with azo dyes. Literature indicates that azo dyes are reduced by bacteria into amines in the intestinal tract of man (Chung et al., 1992; Rafii et al., 1995). The amines are transported well across the intestinal epithelium. Therefore, as a worst case, we assume that all azo dyes that are mobilised from the textile into the saliva, contribute to internal exposure. Hence, the in vitro digestion model was restricted to the saliva phase rather than the entire gastro-intestinal system.

For the experiments with azo dyes in textile, the saliva phase of the suck model is used. Further details about the suck procedure are provided by Oomen et al. (Oomen et al., 2003). In short, the digestion starts by introducing 21 ml of stimulated saliva (pH 6.8) to 0.04-0.4 g of textile. This mixture is rotated head-over-heels for 7.5-60 min at 55 rpm at 37 °C. Subsequently, the saliva is centrifuged during 5 min at 2750*g*, and the supernatant is transferred into a new tube. Analysis is performed by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren) by GC-MS after solvent extraction with dichloromethane.

4.3 Experiments

The experiments with azo dyes were limited due to the amounts of available textile (± 8 g of each textile). Therefore, it was decided to use amounts of 0.4 g textile per digestion only in a few occasions without replicas. Digestions with 0.04 and 0.1 g textile were preferred and these experiments were performed in triplo.
The experiments were designed to investigate the variables listed below. The rationale for the variables is addressed.

Suck time. The duration of the suck phase was varied between 7.5 and 60 minutes. The aim of varying the suck time is to be able to determine an extraction rate, i.e. an amount of dye that is mobilised from textile per min, that can be implemented in the human exposure model ConsExpo (Van Veen, 2001). In addition, insight into the time profile of release of azo dye is obtained.
The duration of the suck time was varied between 7.5 and 60 min as these are normal mouthing times for young children (Groot et al., 1998; Juberg et al., 2001; Bremmer et al., 2002).
Repeated sucking. Pieces of textile were digested, and after removal of the saliva, the textile pieces were left in the test tube to dry, and the same pieces of textile were digested for a second and third time. In this manner, a situation is simulated that a child sucks multiple times on the same piece of textile.
Amount of textile. The amount of textile was varied between 0.04 g and 0.4 g per digestion tube. This was done in order to assess whether the amount of dye released per kg textile was constant, or that saliva was saturated with the compound.
Filtration of saliva. In a few cases the saliva was filtered across paper filters (Machery-Nagel 617 ¼ 18.5 cm, cat no. 534018), to remove small textile fibres that might be released from the textile during digestion. In this manner it was investigated whether the azo dye was solubilised in the saliva, or sorbed to textile fibres suspended in the saliva.
Washing of textile before digestion. A small amount of textile was washed in a washing machine. Part of the washed textile was used for total analysis of amines without in vitro digestion. Another part was digested and the saliva was analysed. In this manner, the effect of washing was investigated. Together with the experiments on repeated sucking and filtration of the saliva, this experiment gives insight into the ability of the azo dye to remain sorbed to the textile or to dissolve into aqueous liquid.
Water versus saliva. Previous research by task group 2 of working group 9 of the European Committee for Standardisation (CEN) showed that the mobilisation of several compounds from plastic films did not differ between saliva and water (Hillersborg, 2002). To study whether the same conclusion holds for azo dyes, the bioaccessibility of aniline, 2,4-toluenediamine, and o-dianisidine is compared after digestion with water and saliva. Furthermore, the effect of filtering water and saliva is compared.

For the present bioaccessibility calculation, it is assumed that the azo dye in the textile releases one aromatic amine after reduction. In principle, one molecule of the azo dye may release one, two, or more amines after reduction. Several amines per molecule of azo dye would lead to a proportional lower bioaccessibility of the dye. Because the number of amines released by the dye is unknown and because toxicity is based on the aromatic amines, the bioaccessibility calculation is based on the aromatic amines.

4.4 Results and discussion

4.4.1 Aniline

4.4.1.1 Analysis

Analysis of aniline was performed by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren). 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 was 1.0 ng per ml of extraction fluid, corresponding to a bioaccessibility of 0.01% for a digestion with 0.1 g of textile.

4.4.1.2 Suck time and repeated sucking

Figure 5 presents the effects of increasing the suck time from 7.5 to 60 min. Experiments were performed with 0.1 and 0.4 g of textile. With both amounts of textile 3 subsequent digestions were performed with the same pieces of textile, which is shown as digestion 1 to 3. As can be seen for each digestion, the bioaccessibility is about constant between 7.5 and 60 min. It can thus be concluded that the same percentage of aniline is released from textile, irrespective of the suck time.

Figure 5 also displays the effect of repeated sucking. Digestion 2 and 3 represent the bioaccessibility of aniline after digesting the same pieces of textile for the second and third time, respectively. As can be seen, the bioaccessibility after the first digestion amounts approximately 7-8%, which corresponds to a release of 105-120 μg aniline per g of textile. The bioaccessibility of aniline after the second and third time drops to approximately 1-2%, corresponding to 12-24 μg/g. This indicates that part of the azo dye that after reduction produces the amine aniline, is weakly bound to the textile, and is mobilised from the textile into the saliva shortly after contact. When this weakly bound part of the azo dye is removed after the first digestion, the mobilisation into saliva decreases substantially to 1-2%. This suggests that a maximum amount of aniline can be released from the same piece of textile after multiple sucking events. In the present experiments the sucking with same pieces of textile was repeated only three times, so that extrapolation to sustained use is difficult. However, it can be assumed that probably less than 20% of the aniline can be released after multiple sucking events, i.e. 8% + 2% + 1% + <9%, with the latter value the extrapolated percentage of aniline that can be released when sucking for the forth and more times on the same piece of textile. The maximum release of aniline from textile of <20% indicates that a child is exposed to a fraction of the total amount of azo dye that is present in the textile.

Note that the bioaccessibility is calculated using the amount of aniline that was released after reduction of untreated textile as 100% (1510 mg/kg). No correction was made in the calculation of the bioaccessibility (%) for the decrease in the amount of aniline in textile due to previous digestions.

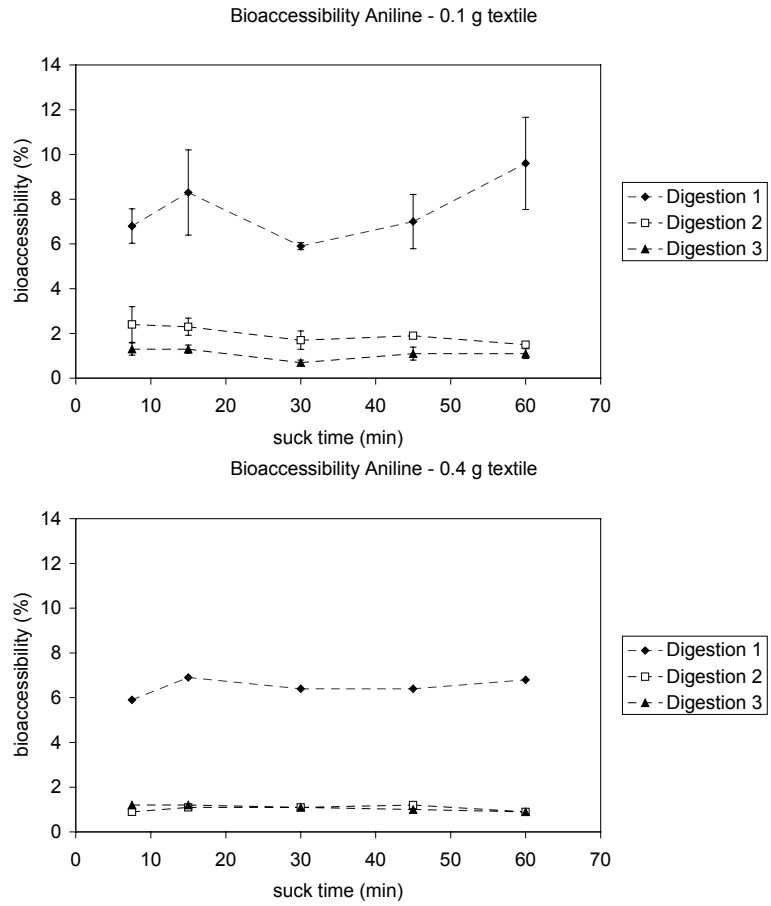

Figure 5. Bioaccessibility of aniline after digestion of 0.1 g and 0.4 g textile, after increasing sucking time.

Digestion 1, 2, and 3 represent in vitro digestion with the same pieces of textile for the first, second and third time. Between digestions, the textile was separated from the saliva simulant and left to dry in the test tube. Data are mean ± SD of 3 digestions with 0.1 g of textile, whereas single digestions were performed with 0.4 g of material.

4.4.1.3 Amount of textile

The effect of the amount of textile on the bioaccessibility of aniline is presented in Table 13, and graphically in Figure 6. Since it was already concluded that the incubation time did not affect bioaccessibility, the bioaccessibility of various incubation times were combined in Figure 6. It can be concluded from Table 13 and Figure 6 that the bioaccessibility of aniline ranged between 6-10%, with an average of 8%, and was not affected by the amount of textile per digestion tube. In other words, the percentage of aniline released from the textile during saliva digestion per kg textile was the same, but the absolute amount of aniline in saliva increased dose proportional with the amount of textile.

Table 13. Bioaccessibility (%) of aniline after in vitro digestion of textile with saliva simulant.

Duration sucking phase (min)	Amount of textile (g)		
	0.04 g	0.10 g	0.4 g
7.5		6.8 ± 0.8	5.9
15		8.3 ± 1.9	6.9
30	8.6 ± 1.1	5.9 ± 0.2 9.8 ± 2.0	6.4
45		7.0 ± 1.2	6.4
60	7.2 ± 0.8	9.6 ± 2.1 7.4 ± 1.5	6.8

Data are of first digestions with a certain piece of textile, and are mean ± SD of 3 digestions, except for data of 0.4 g textile where n=1.

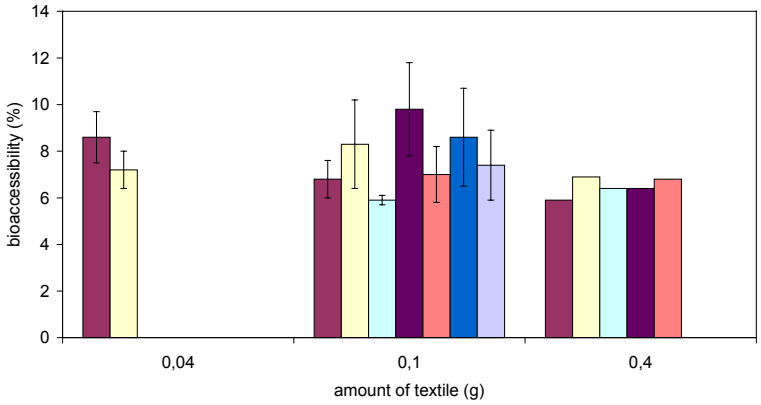

Figure 6. Bioaccessibility of aniline for 0.04, 0.1, and 0.4 g of textile.
The different bars per textile amount represent experiments with different incubation times or the same experiment on various days. Data of first digestions for a certain piece of textile, and are mean ± SD of 3 digestions, except for data of 0.4 g textile where n=1.

4.4.1.4 Filtration of saliva and textile washing

After in vitro digestion, small textile fibres were visible in the saliva simulant. By filtering the saliva over a paper filter, it was investigated whether the azo dye was bound to textile fibres or solved in saliva simulant. The bioaccessibility of aniline was slightly decreased after filtration, but this effect was not significant, see Figure 7. Apparently, azo dye producing the amine aniline was mainly present in the aqueous solution.
The bioaccessibility of aniline after mechanical washing the textile was reduced from about 8% to about 1%, see Figure 7. This is in agreement with the results of repeated digestions with the same pieces of textile. From these experiments, it was concluded that probably part of the azo dye was weakly bound to the textile, and was mobilised from the textile into the saliva shortly after contact. This weakly bound part of the azo dye was also mobilised during washing of the textile, and did not contribute to the bioaccessibility of aniline, resulting in the low bioaccessibility of approximately 1%. Hence, it is concluded that the maximum percentage of aniline that can be released from washed textile after multi sucking events is decreased considerably compared to untreated textile.

After washing, the amount of aniline from undigested textile was determined by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren). A decrease of about 7% in the total amount of aniline determined from untreated and washed textile is expected, i.e. similar to the difference in bioaccessibility after in vitro digestion with untreated textile and washed textile. However, the amount of aniline was 1586 mg/kg, whereas the amount of aniline determined from untreated, undigested textile was 1510 mg/kg (see Table 12). The results can be explained by experimental variability, as in both cases only one textile sample was analysed.

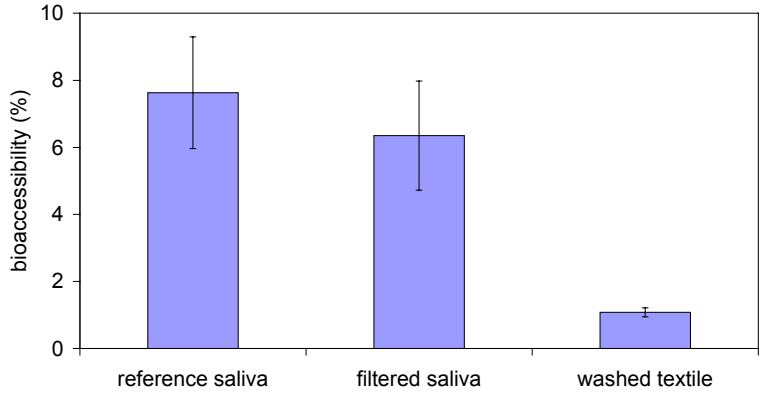

Figure 7. Effect of filtration of saliva and washing of textile before in vitro digestion on the bioaccessibility of aniline.
The data of reference saliva represents a mean ± SD of all bioaccessibility measurements for aniline with saliva (n=32), i.e. including all first digestions with various incubation times and amounts of textile. Other data are mean ± SD of 3 digestions (washed textile) or 2 digestions (filtered textile).

4.4.1.5 Water versus saliva

As can be seen from Figure 8, there is no significant difference between the bioaccessibility of aniline from textile after digestion with water (30 or 60 min incubation, and 0.04 or 0.10 g of textile), compared to the bioaccessibility after digestion with saliva simulant.
Similar to the filtration of saliva (§ 4.4.2.4), filtration of water did not decrease the bioaccessibility of aniline.

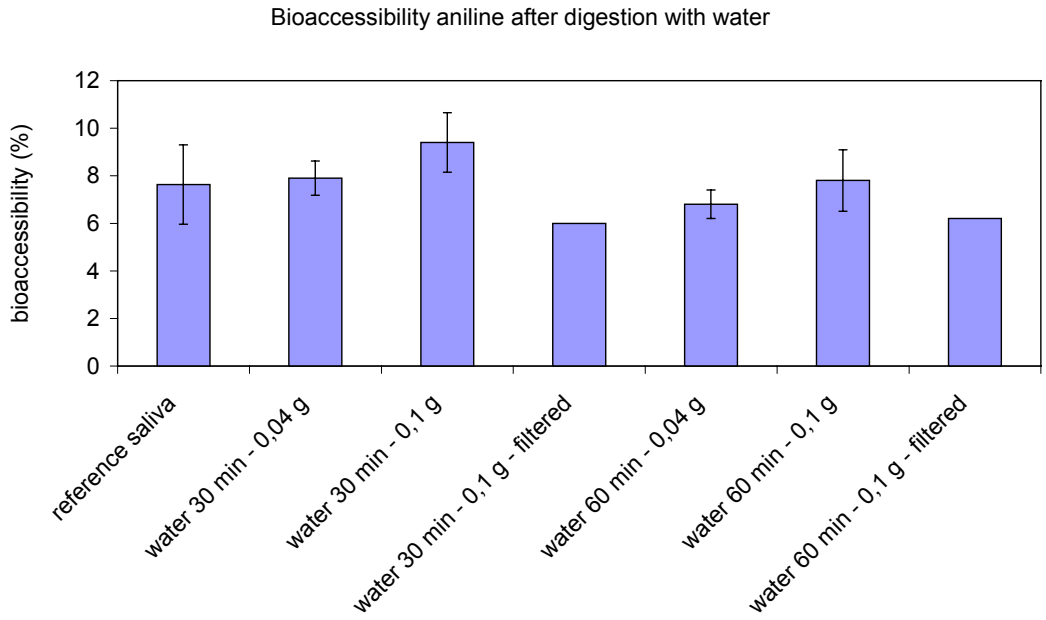

Figure 8. Difference in bioaccessibility of aniline after in vitro digestion with saliva and water.
The data of reference saliva represents a mean ± SD of many bioaccessibility measurements for aniline with saliva (n=32), i.e. including all first digestions with various incubation times and amounts of textile. Other data are mean ± SD of 3 digestions, except for filtered samples where n=1.

4.4.1.6 Conclusions Aniline

Suck time. Bioaccessibility of aniline did not increase with increasing digestion time.
Repeated sucking. The bioaccessibility of aniline after in vitro digestion with saliva simulant was approximately 8% after a first digestion, 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 was weakly bound and was released shortly after contact with saliva or water. When this weakly bound azo dye was removed the bioaccessibility decreased considerably. Although indicative due to the small number of repeated sucking events, it can be assumed that probably less than 20% of aniline can be released from the textile by multiple sucking events.
Amount of textile. The percentage of aniline that became bioaccessible remained the same when digesting different amounts of textile, that is, the absolute amount of bioaccessible aniline increased in proportion with the amount of textile.
Filtration of saliva. Filtration of saliva did not result in a large decrease in the determined concentration of aniline, suggesting the azo dye was not sorbed to textile fibres.
Washing of textile before digestion. The bioaccessibility of aniline after digestion of textile that had been washed in a washing machine was approximately 1%. This is in agreement with the results of repeated sucking, where bioaccessibility was also reduced to 1-2% when the same pieces of textile were digested a second or third time. Based on these bioaccessibility values, it is concluded that the maximum amount of aniline that can be released from washed textile by multiple sucking events was decreased considerably compared to untreated textile.
Water versus saliva. Extraction with water instead of saliva simulant did not result in significantly different bioaccessibility of aniline.

General remark. It should be noted that other azo dyes producing the same aromatic amine after reduction (aniline) may show different behaviour. In addition, different types of textile have different textile properties, which may also lead to different bioaccessibility values. Yet, the present study indicates that it is likely that only a fraction of the azo dye is actually released from textile during sucking.

4.4.2 2.4-toluenediamine

4.4.2.1 Analysis

Analysis of 2,4-toluenediamine was performed by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren). The correlation coefficient of the calibration curve was 0.995, with an average recovery of 105.7% and a relative standard deviation of 11.5%. The limit of quantification was 5.0 ng per ml of extraction fluid, corresponding to a bioaccessibility of 0.1% for a digestion with 0.1 g of textile.

4.4.2.2 Suck time and repeated sucking

Figure 9 presents the effects of increasing the suck time from 7.5 to 60 min for 2,4-toluenediamine. Experiments were performed with 0.1 and 0.4 g of textile. With both amounts of textile, three series of digestions were performed with the same pieces of textile, which are shown as digestion 1 to 3. As can be seen for each digestion, the bioaccessibility ranges between 2 and 10%. This corresponds to a release of 10-50 μg 2,4-toluenediamine per g textile. This indicates that only a small fraction of the azo dye is released from textile by sucking once on the textile by a child.
Digestions with 0.4 g of textile might show a slight, but not significant, increase in bioaccessibility with increasing duration of the saliva phase, whereas the bioaccessibility of 2,4-toluenediamine remains constant for digestions with 0.1 g of textile. Therefore, it is assumed that bioaccessibility of 2,4-toluenediamine is constant in time.

Figure 9 also displays the effect of repeated sucking. Digestion 2 and 3 represent the bioaccessibility of 2,4-toluenediamine after digesting the same pieces of textile for the second and third time, respectively. As can be seen, bioaccessibility does not differ significantly between the first digestion versus the second and third digestion time. This suggests that the same amount of azo dye that produces the amine 2,4-toluenediamine after reduction is released from textile, irrespective of whether the piece of textile has been sucked on before or not.

Note that the bioaccessibility is calculated using the amount of aniline that was released after reduction of untreated textile as 100% (1510 mg/kg). No correction was made in the calculation of the bioaccessibility (%) for the decrease in the amount of aniline in textile due to previous digestions.

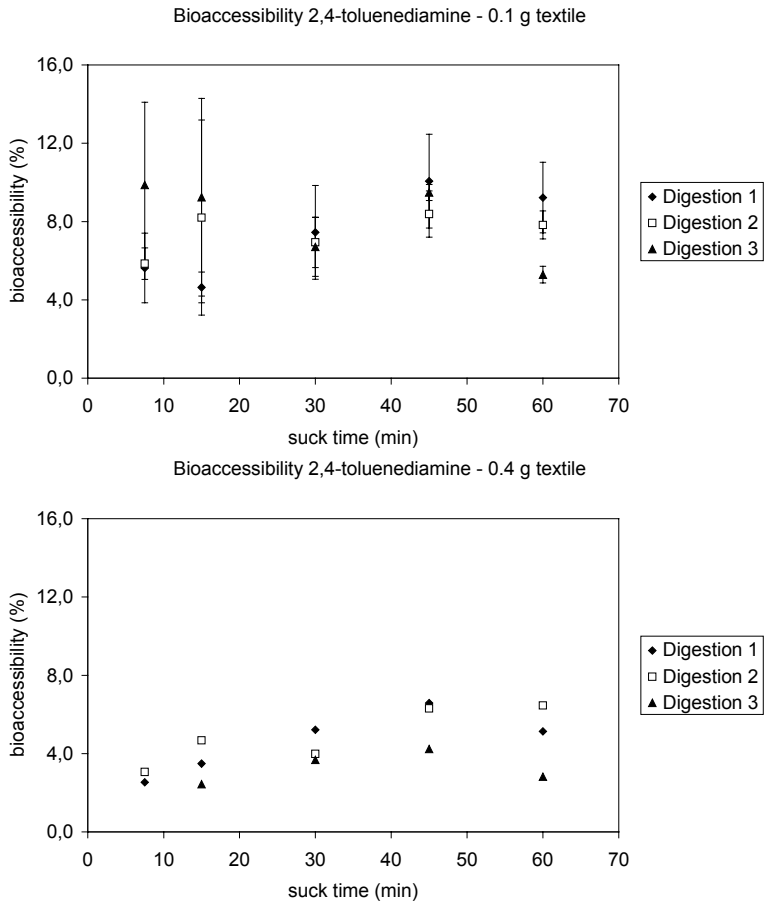

Figure 9. Bioaccessibility of 2,4-toluenediamine after digestion of 0.1 g and 0.4 g textile, after increasing sucking time.

Digestion 1, 2, and 3 represent in vitro digestion with the same pieces of textile for the first, second and third time. Between digestions, the textile was separated from the saliva simulant and left to dry in the test tube. Data are mean ± SD of 3 digestions with 0.1 g of textile, whereas single digestions were performed with 0.4 g of material.

4.4.2.3 Amount of textile

The effect of the amount of textile on the bioaccessibility of 2,4-toluenediamine is presented in Table 14. It can be concluded from Table 14 that the bioaccessibility (%) varies between 2 and 10%, and is not affected by the amount of textile per digestion tube. In other words, the amount of 2,4-toluenediamine released from the textile during saliva digestion per kg textile is the same.

The average bioaccessibility of 2,4-toluenediamine is 6.8 ± 3.0%, when including the data of different suck times, the repeated digestions and different amounts of textile (n=66). This corresponds to a release of 34 μg/g textile.

Table 14. Effect of the amount of textile on the bioaccessibility of 2,4-toluenediamine in saliva simulant.

Duration sucking phase (min)	Amount of textile (g)		
	0.04 g	0.10 g	0.4 g
7.5		5.6 ± 1.8	2.5
15		4.6 ± 0.8	3.5
30	5.6 ± 2.5	7.4 ± 2.4 5.5 ± 2.2	5.2
45		10.1 ± 2.4	6.6
60	9.3 ± 5.5	9.2 ± 1.8 6.1 ± 4.8	5.1

Data are mean ± SD of 3 digestions, except for digestions with 0.4 g of textile, in which case n=1. Only values after a single digestion of a piece of textile are presented.

4.4.2.4 Filtration of saliva and textile washing

The bioaccessibility of 2,4-toluenediamine was decreased considerably after filtration (0.4-1.3%), see Figure 10. Apparently, azo dye producing the amine 2,4-toluenediamine after reduction was sorbed to textile fibres. In contrast to azo dyes that are solubilised in saliva, not necessarily all of the present azo dye that is swallowed is released from the textile during passage in the gastrointestinal tract, as the binding between azo dye and textile is apparently relatively strong. Therefore, it is recommended to determine the bioaccessibility of this azo dye after simulation of digestion of the textile in the gastro-intestinal tract, i.e. including the stomach and intestinal compartment.

The bioaccessibility of 2,4-toluenediamine was lower when textile was washed (3.9%) than without pretreatment (6.8 ± 3.0%), although not significant. Fibres may still be released from the washed pieces of textile, perhaps to a lower extent than from unwashed textile, possibly leading to a somewhat lower bioaccessibility of 2,4-toluenediamine of the washed textile. Yet, the difference in bioaccessibility before and after washing is not large and may also be explained by experimental variation.

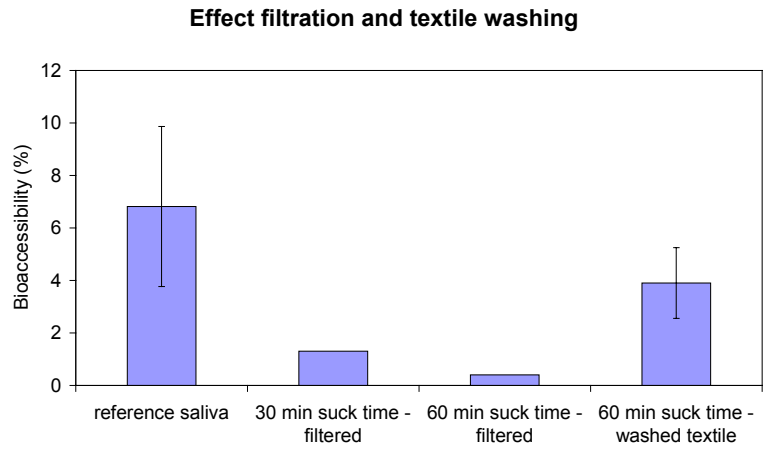

Figure 10. Effect of filtration of saliva and mechanical washing of textile before in vitro digestion on the bioaccessibility of 2,4-toluenediamine.

The data of reference saliva represents a mean ± SD of all bioaccessibility measurements with saliva (n=66), i.e. including the data of different suck times, the repeated digestions and different amounts of textile. Data of washed textile are mean ± SD of 3 digestions, data of filtered saliva are based on one measurement.

The concentration of 2,4-toluenediamine was determined by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren) in untreated textile and in washed textile. The concentration was respectively 499 mg/kg and 437 mg/kg. The slight decrease in total 2,4-toluenediamine concentration between the untreated and washed textile is in agreement with the low bioaccessibility of 2,4-toluenediamine in saliva.

4.4.2.5 Water versus saliva

As can be seen from Table 15, the difference in bioaccessibility of 2,4-toluenediamine after digestion with saliva simulant or water is not large, and due to the high variation, in most cases not significant. However, in all cases the bioaccessibility in saliva is higher than in water, suggesting that saliva is a slightly better extraction fluid than water.
Similar to the filtration of saliva (§ 4.4.3.4), filtration of water resulted in a considerable decrease in bioaccessibility, again indicating that 2,4-toluenediamine is sorbed to textile fibres.

Table 15. Difference in bioaccessibility of 2,4-toluenediamine after in vitro digestion with saliva simulant or water.

Amount of textile (g)	Duration saliva phase (min)	Other conditions	Bioaccessibility in saliva (%)	Bioaccessibility in water (%)
0.04	30		5.6 ± 2.5	4.0 ± 2.5
0.1	30		7.4 ± 2.4 5.5 ± 2.2	3.3 ± 1.8
0.1	30	Filtered	1.3	1.0
0.04	60		9.3 ± 5.5	5.4 ± 2.5
0.1	60		9.2 ± 1.8 6.1 ± 4.8	4.2 ± 1.3
0.1	60	Filtered	0.4	1.1

4.4.2.6 Conclusions 2,4-toluenediamine

Suck time. Bioaccessibility of 2,4-toluenediamine did not increase with increasing digestion time.
Repeated sucking. The bioaccessibility of 2,4-toluenediamine after in vitro digestion with saliva simulant varied between 2 and 10%, with an average of 6.8 ± 3.0%, which corresponds to a release of 34 µg 2,4-toluenediamine per g textile. Bioaccessibility did not differ between the first, second, or third digestion with the same pieces of textile. This suggests that the same amount of azo dye that produces the amine 2,4-toluenediamine after reduction was released from textile, irrespective of whether the piece of textile has been sucked on before or not.
Amount of textile. The percentage of 2,4-toluenediamine that became bioaccessible when different amounts of textile where used remained the same, that is, the absolute amount of bioaccessible 2,4-toluenediamine increased in proportion with the amount of textile.
Filtration of saliva. Filtration of saliva resulted in a considerable decrease in bioaccessibility (0.4-1.3% versus 6.5-7.6%), suggesting the azo dye that produced the amine 2,4-toluenediamine after reduction was sorbed to textile fibres in the saliva. It should be noted that not necessarily all of the present azo dye that is swallowed is released from the textile during passage in the gastrointestinal tract. Therefore, it is recommended to determine the

bioaccessibility of this azo dye after simulation of digestion in the gastro-intestinal tract, i.e. including the stomach and intestinal compartment.
Washing of textile before digestion. Bioaccessibility of 2,4-toluenediamine after digestion of textile that had been washed in a washing machine was 3.9%, which is somewhat lower but not significantly lower than the bioaccessibility of textile that had not been washed (6.8 ± 3.0%). Possibly, less textile fibres were released from textile that had been washed than from untreated textile.
Water versus saliva. Extraction with water resulted in all cases in a lower bioaccessibility than when extraction was performed with saliva simulant, though the difference was in most cases not significant. This suggests that saliva simulant was a slightly better extraction fluid than water.
General remark. It should be noted that other azo dyes producing the same aromatic amine after reduction (2,4-toluenediamine) may show different behaviour. In addition, different types of textile have different textile properties, which may also lead to different bioaccessibility values. Yet, the present study indicates that it is likely that only a fraction of the azo dye is actually released from textile during a single suck event.

4.4.3 o-Dianisidine

4.4.3.1 Analysis

Analysis of o-dianisidine was performed by the Inspectorate for Health Protection (NL: Keuringsdienst van Waren). The calibration curve shows a quadratic relationship with a correlation coefficient of 0.996, an average recovery of 89.3% and a relative standard deviation of 13.3%. Most of the samples of the first series of experiments, including the comparison between saliva and water and the samples after filtration, were lower than the lowest calibration point. Therefore, the calibration curve was extrapolated, leading to a greater uncertainty.
One or two lower calibration points were included for the subsequent series of experiments, i.e. digestion 1 till 3 (see Figure 11). Some samples were still below the lowest calibration point for digestion 1. After including of the second lower calibration point, samples of digestion 2 and 3 were all within the range of the calibration curve.
The limit of quantification was 5-20 ng/ml, depending on the series of analysis, which corresponds with a bioaccessibility of 0.07-0.3% for digestion with 0.1 g of textile.

4.4.3.2 Suck time and repeated sucking

Figure 11 presents the effects of increasing the suck time from 7.5 to 60 min for o-dianisidine. Experiments were performed with 0.1 and 0.4 g of textile. With both amounts of textile, three series of digestions were performed with the same pieces of textile, which are shown as digestion 1 to 3. As can be seen for each digestion, the bioaccessibility ranged between 0.2 and 1.8% for digestions with 0.1 g of textile (= 0.14-1.3 μg per 0.1 g textile), and between 0.04 and 0.38% for digestions with 0.4 g of textile (= 0.11-1.1 μg per 0.4 g textile). This indicates that only a very small fraction of the azo dye is released from textile during sucking once on the textile by a child.
For digestion 1 with 0.1 g of textile, the bioaccessibility of o-dianisidine appears to increase with increasing suck time. However, digestion 2 and 3 with 0.1 g of textile and all digestions with 0.4 g of textile do no show this relationship. Hence, the bioaccessibility of o-dianisidine remains probably constant in time.

Figure 11 also displays the effect of repeated sucking. Digestion 2 and 3 represent the bioaccessibility of o-dianisidine after digesting the same pieces of textile for the second and third time, respectively. As can be seen, bioaccessibility does not differ significantly between the first digestion versus the second and third digestion time, although experimental variation can be large. This suggests that the textile released the same amount of azo dye that produces the amine o-dianisidine after reduction, irrespective of whether the piece of textile had been sucked on before or not. Yet, it should be kept in mind that the concentration of o-dianisidine in the samples was close to the limit of quantification, and extrapolation of the calibration curve was sometimes necessary.

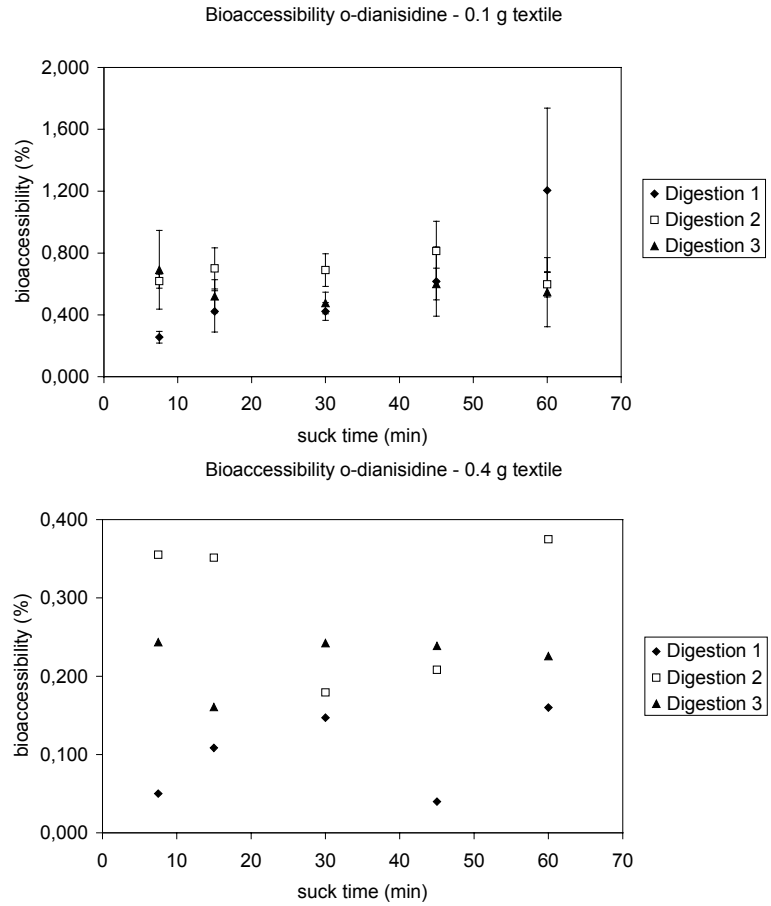

Figure 11. Bioaccessibility of o-dianisidine after digestion of 0.1 g and 0.4 g textile, after increasing sucking time.
Digestion 1, 2, and 3 represent in vitro digestion with the same pieces of textile for the first, second and third time. Between digestions, the textile was separated from the saliva simulant and left to dry in the test tube. Data are mean ± SD of 3 digestions with 0.1 g of textile, whereas single digestions were performed with 0.4 g of material.

4.4.3.3 Amount of textile

The effect of the amount of textile on the bioaccessibility of o-dianisidine is presented in Table 16. Bioaccessibility is on average 0.6 ± 0.3% (n=44) for 0.1 g of textile, and 0.2 ± 0.1% (n=15) for 0.4 g of textile. The data suggest that saliva was saturated with o-dianisidine, as bioaccessibility (%) decreased approximately by a factor 3 from 0.1 g and 0.4 g of textile. The absolute amount of o-dianisidine in saliva simulant was in both cases the

same. The bioaccessibility of o-dianisidine after digestion with 0.04 g of textile was not larger than for 0.1 g of textile, possibly due to the low number of samples and the large uncertainty in the results due to extrapolation of the calibration curve. It is also possible that saturation of the saliva with o-dianisidine was not reached for digestions with 0.04 g of textile.
When assuming saturation of the saliva with o-dianisdine, the saturation level is approximately 18 ng/ml. In this case, bioaccessibility is dependent on the saliva flow rate. Adults have a flow rate of approximately 0.5 ml/min, whereas the maximum flow rate of saliva is reached at the age of 3 to 4 years, which is 8 times higher than the adult basal flow rate (Guyton, 1991; Kedjarune et al., 1997; Navazesh et al., 1992; De Zwart et al., 2002). Assuming a saliva flow rate of 4 ml/min for children and a concentration of o-dianisidine in saliva of 18 ng/ml, an extraction rate of 72 ng/min is deduced during sucking.

Table 16. Effect of the amount of textile on the bioaccessibility (%) of o-dianisidine in saliva.

	Bioaccessibility of o-dianisidine (%)		
Duration sucking phase (min)	Amount of textile (g)		
	0.04 g	0.10 g	0.4 g
7.5		0.56 ± 0.23 (n=8)	0.22 ± 0.15 (n=3)
15		0.55 ± 0.16 (n=9)	0.21 ± 0.13 (n=3)
30	0.41 ± 0.04 (n=2)*	0.52 ± 0.14 (n=12)	0.19 ± 0.05 (n=3)
45		0.68 ± 0.19 (n=9)	0.16 ± 0.11 (n=3)
60	0.34 (n=1)*	0.70 ± 0.43 (n=11)	0.25 ± 0.11 (N=3)

Data are mean ± SD, the number of replicates is indicated by n. Values of three subsequent digestions were used when available.
* Other value(s) below the limit of quantification.

4.4.3.4 Filtration of saliva and textile washing

Digestions were performed with 0.1 g of textile for 30 or 60 min with saliva simulant or water, and subsequently filtered. However, bioaccessibility of o-dianisidine determined after filtration was in all cases below the limit of detection of 5 ng/ml in extraction fluid, which corresponds with a maximum bioaccessibility of 0.08%. It can thus be concluded that filtration lead to a lower bioaccessibility than without filtration (<0.08% versus 0.6-0.3%), so that is seems that the dye was mostly sorbed to textile fibres in saliva. This is not in agreement with saturation of saliva simulant with the azo dye (§ 4.4.3.3). This might be due to the relative large uncertainty in the results. It is also possible that the azo dye producing the amine o-dianisidine after reduction sorbed to the paper filter. In order to gain insight into the behaviour of o-dianisidine in saliva, we recommend to study saturation of o-dianisidine in saliva by incubation of the textile pieces in various volumes of saliva simulant.
The bioaccessibility of o-dianisidine after digestion (60 min) with saliva simulant of 0.1 g washed textile was 0.31 ± 0.14% (n=2). This is lower than the average bioaccessibility of o-dianisidine of 0.1 g untreated textile (0.6 ± 0.3%), but not significantly different.

4.4.3.5 Water versus saliva

As can be seen from Table 17, the difference in bioaccessibility of o-dianisidine after digestion with saliva simulant or water is not large, and in most cases not significant.

Table 17. Difference in bioaccessibility of o-dianisidine after in vitro digestion with saliva simulant or water.

Amount of textile (g)	Duration saliva phase (min)	Bioaccessibility in saliva (%)	Bioaccessibility in water (%)
0.04	30	0.41 ± 0.04 (n=2)*	0.39 ± 0.09 (n=3)
0.1	30	0.52 ± 0.14 (n=12)	0.21 ± 0.03 (n=3)
0.04	60	0.34 (n=1)*	0.31 (n=1)*
0.1	60	0.70 ± 0.43 (n=11)	0.34 ± 0.12 (n=3)

The number of replicates is indicated by n.
* Other sample(s) were below limit of quantification of 5 ng/ml, corresponding with 0.2% bioaccessibility.

4.4.3.6 Conclusions o-dianisidine

Suck time. Bioaccessibility of o-dianisidine from textile was probably constant with digestion time.

Repeated sucking. Bioaccessibility of o-dianisidine after in vitro digestion with saliva simulant was on average 0.6 ± 0.3% (0.1 g of textile), which corresponds with a release of 0.43 µg o-dianisidine per 0.1 g of textile. This indicates that only a very small fraction of the azo dye is released from textile when children suck once on contaminated textile. Sucking a second or third time on the same pieces of textile did not result in a decrease in bioaccessibility of o-dianisidine.

Amount of textile. The percentage of o-dianisidine decreased nearly proportionally with increasing the amount of textile from 0.1 to 0.4 g, that is, the absolute amount of bioaccessible o-dianisidine remained similar. This suggests that the saliva was saturated with o-dianisidine (± 18 ng/ml saliva). In case of saturation, a release rate of o-dianisidine of 72 ng/min can be deduced based on saliva flow rate in children.

Filtration of saliva. Filtration of saliva resulted in a decrease in bioaccessibility (<0.08% versus 0.6 ± 0.3%), suggesting the o-dianisidine was partly sorbed to textile fibres in the saliva. Saturation of the saliva with o-dianisidine is not expected when o-dianisidine is mainly sorbed to textile fibres. These contradicting results may be due to the relative large uncertainty in the results, or to sorption of o-dianisidine to the paper filter. To ensure that o-dianisidine is saturated in saliva, we recommend incubation of textile pieces in various volumes of saliva simulant.

Washing of textile before digestion. The bioaccessibility of o-dianisidine after digestion (60 min) with saliva simulant of 0.1 g washed textile was 0.31 ± 0.14% (n=2). This is lower than the average bioaccessibility of o-dianisidine of 0.1 g untreated textile (0.6 ± 0.3%), but not significantly different.

Water versus saliva. Extraction with water instead of saliva simulant resulted in similar bioaccessibility values.

General remark. It should be noted that other azo dyes producing the same aromatic amine after reduction (o-dianisidine) may show different behaviour. In addition, different types of textile have different textile properties, which may also lead to different bioaccessibility values. Yet, the present study indicates that it is likely that only a fraction of the azo dye is actually released from textile during a single suck event.

4.5 Comparison of the bioaccessibility of 2,4-toluenediamine, aniline, and o-dianisidine

The experimental variations that were performed with the three textiles in order to investigate the behaviour of the azo dyes are presently compared.

Suck time. All aromatic amines showed a constant bioaccessibility (%) in time between 7.5 and 60 minutes suck time. Hence, for none of the presently studied amines, a release rate in saliva could be determined from the experiments with increasing suck time. Apparently, the transfer of aromatic amine to saliva simulant was fast (< 7.5 min).

Repeated sucking. Of the presently studied amines, the bioaccessibility of aniline was considerably higher for untreated textile (8%) than for textile that had been digested before in the model (1-2%). This indicates that part of the azo dye was weakly bound to the textile and was released shortly after contact with water. The other two amines did not show this behaviour.

Amount of textile. For aniline and 2,4-toluenediamine, the bioaccessibility (%) was constant for different amounts of textile, varying between 0.04 and 0.4 g per digestion. In other words, the absolute amount of bioaccessible amine increased in proportion with the amount of textile. The bioaccessibility (%) of o-dianisidine decreased almost proportionally when increasing the amount of textile per digestion from 0.1 to 0.4 g. This suggests that saliva simulant got saturated with o-dianisidine.

Filtration of saliva. Filtration of saliva indicates that aniline was not sorbed to fibres of the presently used textile, whereas 2,4-toluenediamine was mainly sorbed to the textile fibres. The results of o-dianisidine suggest that this amine was also sorbed to textile fibres, although it is also possible that this was due to an experimental artefact, e.g. sorption of the azo dye to the filter during filtration of the saliva simulant.

Washing the textile before digestion. Mechanically washing textile lead to a considerable reduction of the bioaccessibility of aniline (8% for untreated textile versus 1% for washed textile). This is in agreement with the results of aniline for repeated sucking. Hence, washing the present textile before use would be a simple manner to reduce the exposure to aniline considerably. Bioaccessibility of 2,4-toluenediamine and o-dianisidine were lower after digestion of textile that had been washed, but not significantly lower. Possibly, less textile fibres were released from textile that had been washed than from untreated textile.

Water versus saliva. The bioaccessibility of the present amines after in vitro digestion with saliva simulant did not differ significantly from experiments with water as extracting fluid. Yet, the bioaccessibility of 2,4-toluenediamine was in all cases higher in saliva than in water, suggesting that saliva was a slightly better extracting fluid than water.

The experimental variations addressed above indicate that the three azo dyes showed very different behaviour when the contaminated textiles were subjected to simulated sucking.

4.6 Risk assessment

4.6.1 Application to ConsExpo

The presently obtained bioaccessibility data can be used to refine default parameters for leaching in the fact sheet toys that is used by the exposure model ConsExpo (Bremmer et al., 2002). The experiments show that bioaccessibility varies for the different aromatic amines. As a consequence, a default bioaccessibility value for azo dye from textile cannot be determined. Bioaccessibility probably depends both on textile type and azo dye. The results of 2,4-toluenediamine indicate that the azo dye producing 2,4-toluenediamine after reduction was sorbed to textile fibres in saliva simulant. Hence, textile types that release many fibres during sucking will lead to higher bioaccessibility values than textile types that hardly release fibres, e.g. a different bioaccessibility is expected for the same azo dye contamination from wool, cotton or silk. Differences in bioaccessibility between different azo dyes are expected because the presently tested dyes show very different behaviour in sorption to textile fibres and in saturation of the saliva simulant. Because, based on the present data, the effect of the textile type and the azo dye on bioaccessibility cannot be extrapolated to other combinations of textile and azo dye, experimental determination of the bioaccessibility is recommended for future cases of azo dye contaminated textile. When more data become available, relationships can be made between bioaccessibility, textile type, and azo dye/aromatic amine, which subsequently can be implemented in the fact sheets of ConsExpo.
From the present results it appeared that bioaccessibility did not differ significantly between digestion with saliva simulant and water. Therefore, no large differences in bioaccessibility of aromatic amines are expected between extractions with different aqueous solutions. This suggests that release of azo dye from textile into other aqueous solutions than the present saliva simulant can also be used as input for the fact sheets of ConsExpo.

4.6.2 Case studies in cancer risk assessment

Zeilmaker et al. made an assessment of the cancer risk of aromatic amines by elaborating on several case studies (Zeilmaker et al., 2000; Zeilmaker et al., 1999). In one of these case studies the cancer risk associated with oral exposure to 2,4-toluenediamine (359 μg/g) in textile toys was assessed (Zeilmaker et al., 2000). In another case study the cancer risk was associated with oral exposure to o-dianisidine (337 μg/g) in a string of a children's sweater was assessed (Zeilmaker et al., 1999). The assumptions on the leaching of these aromatic amines from the textile products are compared to the results of the present experiments with 2,4-toluenediamine and o-dianisidine.

2,4-Toluenediamine. It was assumed by Zeilmaker et al. that the fraction of 2,4-toluenediamine that migrated out of the textile toys during sustained use was 1, as leaching of 2,4-toluenediamine had not been determined experimentally (Zeilmaker et al., 2000). The present experiments indicate that approximately 7% of 2,4-toluenediamine was released from the tested textile during a single suck event. However, no signs of decreasing bioaccessibility were observed for multiple (up to 3 times) suck events with the same pieces of textile. Hence, in case of sustained use of textile it cannot be excluded that ultimately all dye is removed from the product. In that case, the risk assessment of the present textile would result in a similar assumption on migration of 2,4-toluenediamine out of the textile as the risk

assessment by Zeilmaker et al. (Zeilmaker et al., 2000), i.e. all amine would eventually migrate out of the product. When a single or a few suck events are considered, the removal of 2,4-toluenediamine is decreased to 7% per event.
The data indicate that 2,4-toluenediamine was mainly sorbed to textile fibres. This implies that no textile fibres, i.e. no textile product, would be left at a bioaccessibility of 100%. Hence, it is unlikely that the bioaccessibility is 100% for sustained use. Therefore, for better risk assessment of sustained use, more repeated digestions with the same pieces of textile are recommended.

o-Dianisidine. In the case study by Zeilmaker et al., the leaching of o-dianisidine from a string of a children's sweater to sweat simulant was measured experimentally, and assumed to equal the leaching to saliva, i.e. bioaccessibility, and amounted 0.49 μg/g or 0.15% (Zeilmaker et al., 1999). In the experiment with sweat simulant 1 g of textile product was incubated in 100 ml NaCl/phosphate buffer of pH 6.8 at 37 °C during 16 hours (Zeilmaker et al., 1999). The present experiments indicate that on average 0.6 ± 0.3% of o-dianisidine was released into saliva simulant during a single digestion event, which corresponds with an amount of 4.4 ± 1.9 μg/g textile. These values are derived from digestions with 0.1 g of textile with a contamination level of 709 μg/g. At higher levels of textile, saliva probably becomes saturated with o-dianisidine, and bioaccessibility (%) decreases. Hence, it can be concluded that the release of o-dianisidine from textile estimated from the present experiments (0.6%) is in the same order of magnitude as the release of o-dianisidine from a string of a children's sweater assumed by Zeilmaker et al. (0.15%) (Zeilmaker et al., 1999). As it is uncertain if the same azo dye and textile type were considered in both studies, it is unclear whether the similar results obtained in both studies is coincidental.

The case studies addressed above indicate that bioaccessibility results should be used as input in risk assessment. The physiological basis of the in vitro digestion model gives a scientific foundation to exposure data, rather than non-experimentally based "worst case" assumptions. Comparison of bioaccessibility determined in the present experiments to the assumed release of aromatic amines from textile products by Zeilmaker et al. showed similar values (Zeilmaker et al., 2000; Zeilmaker et al., 1999). The similarity was due to the sustained use of the product and to the similarity in release of o-dianisidine to saliva simulant and sweat simulant.

4.7 Overall conclusions azo dyes

The bioaccessibility of the aromatic amines from the present textiles was low, i.e. approximately 8% for aniline, 7% for 2,4-toluenediamine, and 0.6% for o-dianisidine after a single suck event. **This suggests that children are exposed to a fraction (respectively 8%, 7% or 0.6%) of the total amount of amine that can be obtained after reduction of the azo dye, when sucking once on these textiles.**

Different patterns in bioaccessibility were observed between the three aromatic amines.

- Only aniline showed a decrease in bioaccessibility when the same pieces of textile were digested for the second or third time. Hence, for the presently tested textile, a percentage of aniline can be estimated that can be released from textile by multiple sucking events. Probably less than 20% of aniline can be released from textile by multiple sucking events.

Washing the textile considerably decreased the release of aniline from textile. Aniline was not sorbed to textile fibres.

- For 2,4-toluenediamine a distinction should be made between a single suck event (bioaccessibility 7%), and multiple suck events (100% bioaccessibility could not be excluded for a large number of suck events based on the present experiments). The saliva could not get saturated with the azo dye releasing 2,4-toluenediamine after reduction, because the azo dye appeared to be mainly sorbed to textile fibres. The release of fibres from the textile was thus driving the exposure to 2,4-toluenediamine. This also indicates that it is expected that bioaccessibility of the present dye will be less than 100% even for sustained use, as in case of 100% bioaccessibility no textile would be left. Therefore, for better risk assessment of sustained use, more repeated digestions with the same pieces of textile are recommended. In addition, it should be noted that not necessarily all of the present azo dye that is swallowed is released from the textile during passage in the gastrointestinal tract. Therefore, it is also recommended to determine the bioaccessibility of this azo dye after simulation of digestion in the gastro-intestinal tract, i.e. including the stomach and intestinal compartment.
- The bioaccessibility of o-dianisidine was 0.6% for 0.1 g of textile, and was not found to decrease with a second or third digestion. The saliva became probably saturated with o-dianisidine when more textile was digested, although filtration suggests that part of the o-dianisidine was sorbed to textile fibres, which cannot lead to saturation. These contradicting results may be due to experimental variation or the azo dye may have been sorbed to the paper filter during filtration. Experiments with larger and smaller volumes of saliva are recommended to ensure that saliva simulant was saturated with o-dianisidine. When using the saturation level of o-dianisidine in saliva, and the maximum saliva flow rate in children, an extraction rate of 72 ng o-dianisidine per minute can be calculated during sucking.

The presently obtained bioaccessibility data can be used to refine the fact sheets that are used by the exposure model ConsExpo. The present experiments show that bioaccessibility was different for the different aromatic amines. As a consequence, a default bioaccessibility value for azo dye from textile cannot be determined. Bioaccessibility probably depends on textile type and azo dye. **Therefore, experimental determination of the bioaccessibility is recommended for future cases of azo dye contaminated textile.** When more data are available, relationships can be made between bioaccessibility, textile type, and azo dye/aromatic amine, which subsequently can be implemented in the fact sheets of ConsExpo.

The obtained bioaccessibility results were compared with leach values assumed in the cancer risk assessment of 2,4-toluenediamine and o-dianisidine from textile items after oral exposure (Zeilmaker et al., 2000; Zeilmaker et al., 1999). Similar values for migration of the amines out of the textile products would have been derived for the present textiles. This is because no reduction in leaching of 2,4-toluenediamine could be derived from the present experiments for sustained use, because the three times repetitive digestion is not fully representative for sustained use. The experimentally obtained bioaccessibility of o-dianisidine in the present study was similar to the assumptions made for leaching in the risk assessment, because leaching was based on experimentally determined migration into sweat simulant, which appeared to give similar results.

The case studies addressed above confirm that bioaccessibility is a necessary part of the exposure assessment of azo dyes from consumer products. The physiological basis of the in vitro digestion model gives a scientific foundation to exposure data, rather than non-experimentally based assumptions, and is therefore preferred.

5. Overall conclusions

In the present project in vitro digestion models simulating the human gastrointestinal tract are developed in order to estimate the amount of a contaminant that can be released into digestive fluid and becomes available for absorption (Oomen et al., 2003). First when a compound is absorbed, it will contribute to internal exposure and (possible) systemic toxicity.
This report describes application of the in vitro digestion models to several cases. The following cases were investigated: 1) the effect of mouthing behaviour on the bioaccessibility of lead (Pb) from chalk and paint flakes (Chapter 2), 2) release of phthalate (DINP) from PVC disks into saliva simulant (Chapter 3), and 3) release of azo dyes from textile into saliva simulant (Chapter 4). Specific conclusions are presented at the end of Chapter 2, 3 and 4. Presently, the most important issues are discussed.

The experiments show that the release of compounds from a matrix into digestive fluids is not complete. Values between 4 and 53% were found for the bioaccessibility of Pb from paint flakes and chalk in the intestinal compartment. The release of a phthalate (di-isononylphthalate = DINP) from PVC disks into saliva simulant was on average 0.031 ± 0.002% after 60 min of extraction. Finally, the release of aromatic amines/azo dyes from textiles into saliva simulant was approximately 8% for aniline, 7% for 2,4-toluenediamine, and 0.6% for o-dianisidine after a single suck event. This indicates that children/humans that ingest or suck on these items (once or a few times) are not exposed to the entire load of contaminant in the matrix, but only to a fraction.

The experiments with different solid-to-fluid ratios of Pb contaminated paint flakes and chalk indicate that the amount of matrix may have substantial effect on the bioaccessibility. For example, the bioaccessibility of chalk in the intestinal compartment decreased from about 50% to 4% when increasing the amount of matrix from 0.01 to 0.4 g per digestion tube. For the azo dyes in textile the solid-to-fluid ratio was also varied by increasing the amount of textile in saliva from 0.04 to 0.4 g. These experiments showed a constant bioaccessible percentage, except for o-dianisidine where the saliva was probably saturated. Similarly, changing the solid-to-fluid ratio of PVC disks in saliva simulant resulted in a constant bioaccessibility percentage for the release of phthalate (DINP). This indicates that the relationship between bioaccessibility and solid-to-fluid ratio depends on the matrix and compound.

Phthalate (DINP) migration rate obtained with the *in vitro* digestion model of RIVM (3.3 µg/min/disk) was in the same order of magnitude as the average DINP release in saliva of human volunteers (1.4 µg/min/disk), and comparable to the results of 12 other laboratories. Therefore, the *in vitro* digestion model can be used as a tool to estimate the exposure of children to potentially harmful substances by mouthing their toys and childcare articles. To further evaluate the in vitro digestion models, another validation study is recommended that focuses on the entire digestion process, i.e. including the gastric and intestinal compartment.

Migration of DINP from PVC disks into saliva was higher for the saliva simulant (3.3 µg/min) compared to water (1.8 µg/min). Also release of the azo dye producing the amine 2,4-tolueneamine after reduction, was slightly higher after digestion with saliva simulant versus water. This suggests that saliva simulant might, in some cases, be a slightly better extraction fluid than water. Yet, it is expected that in most cases bioaccessibility in

saliva simulant is in the same order of magnitude as when water is employed as extraction fluid. Large differences are expected between bioaccessibility determined with digestive juices in the gastric or intestinal compartment, and water, due to the high concentrations of digestive constituents in gastric and intestinal fluids.

The bioaccessibility results obtained with the in vitro digestion models can be used to refine the fact sheets used by the exposure model ConsExpo, and can be used as input parameters in risk assessment. Because it appeared that bioaccessibility depends on many variables such as matrix, compounds, solid-to-fluid ratio, and because the in vitro digestion model is a relatively simple procedure, it is recommended to determine bioaccessibility experimentally. When sufficient bioaccessibility data become available, relationships between product characteristics and bioaccessibility can be made, which in turn can be used as more general information in the fact sheets of ConsExpo.

The present applications of the in vitro digestion models for toy show that, based on the results up till now, the in vitro digestion models developed in the present project are promising tools for exposure assessment of contaminants in toys.

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