RIVM report 320102001/2003

Development and suitability of in vitro digestion models in assessing bioaccessibility of lead from toy matrices

AG Oomen, K van Twillert, MFA Hofhuis,
CJM Rompelberg, CHM Versantvoort

This investigation has been performed by order and for the account of the Inspectorate for Health Protection and Veterinary Public Health, within the framework of project V/320102, In vitro digestion model food/toy.

RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Contents

Samenvatting

Kinderen kunnen worden blootgesteld aan stoffen afkomstig uit speelgoed als gevolg van sabbelen en eventueel inslikken van (stukjes) speelgoed. Deze stoffen (contaminanten) kunnen schadelijk zijn voor de gezondheid. Om de gezondheid van het kind te beschermen zijn richtlijnen opgesteld die de aanwezigheid van contaminanten boven een bepaald niveau verbieden, of die een grens aangeven voor de migratiesnelheid van de contaminant uit speelgoed. De omstandigheden waaronder de migratiesnelheid wordt bepaald zijn echter vaak niet fysiologisch. Zo wordt de migratiesnelheid voor enkele metalen in vitro bepaald in een sterk zuur milieu (HCl pH 1-1,5), wat kan leiden tot een overschatting van de daadwerkelijke migratiesnelheid. De matrix (krijt, verf, bijtring etc) kan grote invloed hebben op hoeveel contaminant wordt vrijgemaakt uit speelgoed in het maagdarmkanaal. Alleen het vrijgemaakte deel van de contaminant kan terechtkomen in de bloedbaan (interne blootstelling) en kan toxiciteit veroorzaken. In huidige risicoschatting wordt het vrijmaken van de contaminant uit speelgoed niet wordt meegenomen, of wordt bepaald onder niet-fysiologische condities. Daardoor wordt het risico dat kinderen lopen door blootstelling aan contaminanten in speelgoed overschat. Om een beter beeld te krijgen van de daadwerkelijke migratiesnelheid is er vraag naar een eenvoudige in vitro methode, gebaseerd op fysiologie, om het vrijmaken van contaminanten uit speelgoed onder invloed van sabbelen en/of het digestieproces te bepalen.

Het vrijmaken van contaminanten uit speelgoed in het maagdarmkanaal wordt bioaccessibility genoemd. Dit proces kan worden nagebootst met een in vitro digestiemodel. Het gedrag van kinderen, sabbelen en/of inslikken van speelgoed, kan invloed hebben op de bioaccessibility. In het huidige rapport wordt de ontwikkeling beschreven van een drietal fysiologisch gebaseerde in vitro digestiemodellen. Met deze modellen kan de bioaccessibility van contaminanten uit speelgoed worden bepaald onder verschillende omstandigheden:
1) sabbelen aan speelgoed, 2) sabbelen aan speelgoed in combinatie met inslikken van het speelgoed, en 3) inslikken van speelgoed zonder een sabbelfase. Daarnaast is het effect van enkele variabelen zoals speeksel samenstelling, sabbelduur en speekselvolume op bioaccessibility bestudeerd. Doordat alle drie de modellen een mond-, maag- en darmcompartiment hebben kan de bioaccessibility in de darm bepaald worden. Dit is relevant omdat absorptie van stoffen vrijwel uitsluitend in de darm plaatsvindt.

De drie in vitro digestiemodellen zijn getest voor lood (Pb) uit 4 verschillende speelgoedmatrices (stoepkrijt, bordkrijt, vingerverf, verfschilfers). Pb was gekozen omdat de Keuringsdienst van Waren verscheidene malen speelgoed met hoge Pb concentraties was tegengekomen, en omdat enkele speelgoedmatrices met Pb erin aanwezig waren.
De bioaccessibility in het darmcompartiment was het laagst voor het sabbel model (tussen <0,2% en 5,4%), matig voor het sabbel-slik model (tussen 0,1% en 23%), en het hoogst voor het slik model (tussen 1,6% en 51%). De bioaccessibility in speeksel van het sabbelmodel was laag (tussen <0,2% en 8,4%).

De volgende conclusies kunnen op basis van de huidige studie getrokken worden:

- Risicobeoordeling gebaseerd op migratie van Pb uit speelgoed onder zure condities overschat waarschijnlijk de blootstelling, omdat bioaccessibility van Pb in het dunne darm compartiment (waar absorptie plaatsvindt) 2 tot 50-voud lager is dan de bioaccessibility in de maag (waar een zuur milieu heerst, pH 1,6-2,6 voor huidige experimenten).
- Gebaseerd op de onderzoeksresultaten lijken de in vitro digestiemodellen bruikbaar om de bioaccessibility van contaminanten in speelgoed te bepalen als indicator voor orale biobeschikbaarheid van een contaminant. Deze conclusie is gebaseerd op:
    - Bioaccessibility in het darmcompartiment, waar absorptie plaatsvindt, was in alle gevallen aanzienlijk lager dan 100% (tussen 0,1% en 51% voor de huidige experimenten). Dit suggereert dat slechts een deel van Pb in speelgoed kan bijdragen aan interne blootstelling in de mens.
    - Inslikken van speelgoed leidt tot hogere bioaccessibility c.q. orale biobeschikbaarheid van Pb dan alleen sabbelen op speelgoed, waarschijnlijk als gevolg van de lage pH (maagsap) en hoge concentraties complexerende stoffen (maag- en darmsap).
    - De bioaccessibility c.q. orale biobeschikbaarheid van Pb wordt sterk bepaald door de matrix. De bioaccessibility varieerde tot een factor 230 (tussen 0,1% en 23%) binnen één digestiemodel. Stoepkrijt en krijt gaven aanzienlijk lagere bioaccessibilities dan verf en vingerverf. De invloed van de matrix op bioaccessibility was veel belangrijker dan het contaminatieniveau en de manier van contamineren (kunstmatig of ontstaan tijdens het productieproces).
    - De reproduceerbaarheid van het sabbel-slik digestiemodel was onderzocht en bleek acceptabel en in enkele gevallen groot (binnendagvariatie <25% behalve voor erg lage bioaccessibilities, tussendagvariatie in de meeste gevallen ±50%). De reproduceerbaarheid kan waarschijnlijk worden verbeterd als het stellen van de pH in de maag wordt gestandaardiseerd.
- De resultaten die worden verkregen met het in vitro digestiemodel kunnen zowel in de “sabbel” en “inslik” module van het humane blootstellingsmodel CONSEXPO worden geïmplementeerd. Echter, omdat de bioaccessibility sterk matrix afhankelijk is zal de bioaccessibility voor veel speelgoed/contaminant combinaties moeten worden bepaald teneinde in CONSEXPO een bruikbare dataset op te kunnen stellen. Een andere mogelijkheid is dat de bioaccessibility van een contaminant in speelgoed wordt bepaald voor ieder specifiek geval.

Voordat de in vitro digestiemodellen gebruikt kunnen worden in risicobeoordeling moeten de digestiemodellen worden gevalideerd met in vivo data. Toekomstig onderzoek zal zich hierop richten.

Summary

Children can be orally exposed to compounds by chewing, sucking and ingestion of toy (parts). These compounds (contaminants) may cause health problems. To protect children's health guidelines have been formulated that prohibit the presence of contaminants above a certain level, or that prescribe a migration limit of the contaminant from the toy. The experimental conditions for migration do not always simulate the physiologically situation of children. The migration of several metals is determined in vitro in acid (HCl pH 1-1.5), which may result in overestimation of the true migration limit. The matrix (chalk, paint, teething ring) can have profound influence on the release of contaminants from toy in the gastrointestinal tract. Only the released contaminant can reach the blood stream (i.e. internal exposure) and can exert toxicity. In present risk assessment, the release of contaminant from toy is not accounted for, or is determined under non-physiological conditions. As a consequence, the risk that children run due to exposure to contaminants in toys is overestimated. To improve risk assessment there is demand for a simple in vitro method, based on physiology, to release contaminants from toy caused by sucking and/or the digestion process.

Release of contaminants from toy in the gastrointestinal tract is referred to as bioaccessibility. This process can be simulated in an in vitro digestion model. Handling of toy by children like sucking or swallowing, can affect bioaccessibility. In the present report the development of three physiologically based in vitro digestion model is described. With these models the bioaccessibility of contaminants from toys can be assessed for the following situations: 1) sucking on toy, 2) sucking on toy in combination with swallowing of the toy matrix, and 3) swallow of toy matrix without a sucking phase. In addition, the effect of several variables such as saliva composition, duration of the mouthing phase, and volume of saliva on bioaccessibility is studied. Bioaccessibility in the intestine can be estimated because the three digestion models consist of a mouth, stomach and intestinal compartment. This is relevant as absorption of compounds takes almost exclusively place in the intestine.

The three in vitro digestion models were applied for lead (Pb) from 4 toy matrices (chalk for exterior use, chalk, finger-paint, paint flakes). Pb was used as a contaminant because the issue of Pb in toys has been encountered several times by the Inspectorate for Health Protection and Veterinary Public Health (Keuringsdienst van Waren) and several toy matrices with Pb were available.
The bioaccessibility in the intestinal compartment was lowest for the suck model between <0.2% and 5.4%, intermediate for the suck-swallow model between 0.1% and 23%, and highest for the swallow model between 1.6% and 51%. The bioaccessibility in the mouth compartment of the suck model was low (between <0.2 and 8.4%).

The following conclusions can be drawn on basis of the present study:
- Risk assessment based on migration of Pb from toys under acid conditions is likely to overestimate the oral bioavailability as the bioaccessibility of Pb in the small intestinal

compartment (where absorption takes place) was 2- to 50-fold lower than the bioaccessibility of Pb in the stomach (with pH 1.6-2.6 for the present experiments).

- The in vitro digestion models seem to be a useful tool to investigate the bioaccessibility of contaminants from toys, which can be used as an indicator for oral bioavailability of the contaminant in toy. This conclusion is based on:
    - Bioaccessibility was in all cases considerable lower than 100% (between 0.1% and 51% in the present study), suggesting that only part of Pb in toy matrix can contribute to internal exposure in man.
    - Swallowing the toy matrix leads to a higher bioaccessibility c.q. oral bioavailability of Pb than only sucking on the toy. This is probably due to the low pH (gastric juice) and high concentration complexing agents (both stomach and intestinal juice).
    - The bioaccessibility c.q. oral bioavailability of Pb is strongly dependent on the matrix. Bioaccessibility varied up to a factor 230 (between 0.1% and 23%) within one digestion model. Bioaccessibility of chalk for exterior use (stoepkrijt) and chalk was considerably lower than of paint and finger paint. The influence of the matrix on bioaccessibility was more important than the level of contamination and the manner of contamination (artificially contaminated versus production process contaminated). Oral exposure of children to contaminants is thus expected to vary from toy to toy.
    - Reproducibility of the suck-swallow digestion model was investigated and appeared acceptable and in some cases large (within day variation <25% except for the very low bioaccessibilities, between day variation was in most cases ±50%). The reproducibility will probably improve if adjustment of the pH in the stomach compartment is standardised.
- The data obtained with the in vitro digestion model can be implemented in the human exposure model CONSEXPO for both the “mouthing” and “ingestion” module. However, because bioaccessibility is dependent on the matrix, bioaccessibility of many toy/contaminant combinations should be determined if a fact-sheet on this subject is to be useful. Another option is to assess bioaccessibility for each specific case of a toy and contaminant combination.

The in vitro digestion models should be validated with in vivo data before the digestion models can be used in risk assessment. Therefore, future research will focus on validation.

1. Introduction

1.1 General introduction

The behaviour of young children as they pick up consumer products and toys, put them in their mouths, and lick their fingers might pose them harm. Toys may contain contaminants that may affect the health of a child that is playing with a toy. Guidelines have been formulated in order to protect children's health (NEN-EN 71-3; 1999/815/EC; 93/11/EEG, European Toy Directive 88/378/EEC etc). These guidelines prohibit the presence of contaminants in toys above a certain level or consider a migration limit of the contaminant from the toy. However, the experimental conditions for migration do not always simulate the physiologically situation in children. The migration limit for several metals is determined in vitro in acid (HCl pH 1-1.5), which may result in overestimation of the true migration limit. Furthermore, in human exposure models, the possibility that not all contaminants in the toy contribute to the internal exposure is often not considered. As frequently only a fraction of the compound is released from its matrix, and thus only a fraction contributes to the internal exposure, the risk is overestimated. For that reason, there is a demand for simple tools that can estimate the internal exposure of contaminants caused by handling toys by children.

The aim of the project is to contribute to improvement of exposure assessment for children to contaminants in toys by means of developing an experimental tool by which means internal exposure of children to contaminants from toys can be estimated. The tool should be able to give an estimate of internal exposure for specific cases or for general applicability of contaminants in toys. In addition, it would be valuable if results of the tool can be used as input for the human exposure model CONSEXPO (Bremmer and Van Veen 2002; Van Veen 2001).

With an in vitro digestion model based on human physiology, the mobilisation (bioaccessibility) of compounds from their matrix during transit in the gastrointestinal tract can be investigated. Bioaccessibility is considered to be a prerequisite for oral bioavailability and internal exposure (see chapter 2 for further explanation on bioaccessibility, bioavailability, internal exposure and external exposure). Accounting for bioaccessibility can thus lead to a more realistic risk assessment. Especially bioaccessibility in the small intestinal compartment is important as mainly here absorption of compounds takes place. On the other hand, mobilisation of the contaminant may occur in the mouth as well as in the stomach and small intestine. A three step procedure simulating the digestion process in successively the mouth, stomach and intestine accounts for the entire digestion process. Such an in vitro digestion model based on human physiology was already developed to assess the bioaccessibility of contaminants from soil in children (Oomen *et al.* 2003). This model was used as starting point for an in vitro digestion model for toys. In vitro digestion models based on human physiology are expected to give more realistic measures for mobilisation of contaminants from toys than migration tests that are prescribed in current guidelines (NEN-EN 71-3, European Toy Directive 88/378/EEC).

For exposure to contaminants in toys via the oral pathway, three different scenarios can be distinguished: 1) child sucks on a toy and swallows the contaminants released in saliva, 2) child sucks on a toy, swallows not only the contaminants released in saliva but swallows also the contaminated toy matrix, and 3) child swallows the contaminated toy matrix without a sucking phase. When the toy itself is not ingested, only contaminants that are released from the toy in the saliva during chewing and sucking on the toy will enter the gastrointestinal tract and contribute to the exposure of the child. When the toy matrix is ingested not necessarily all contaminants will be released from the toy in the gastrointestinal tract. The contaminants still sorbed to the toy matrix in the gastrointestinal tract can be considered to be unavailable for absorption, and do not contribute to the exposure.

In this report, three variants of the in vitro digestion model were developed, corresponding to the three possible scenarios for the oral pathway of mouthing toys: 1) a suck model, 2) a suck-swallow model, and 3) a swallow model. The three variants of the in vitro digestion model differ mainly from one another in the mouthing phase of the digestion process, i.e. sucking on a toy for a prolonged period of time or swallowing the toy. To that end, first changes in the mouthing phase considering composition of saliva, mouthing time and saliva volume were studied by means of the bioaccessibility of lead (Pb) in saliva from 2 toy matrices. Pb was used as a contaminant because the issue of Pb in toys has been encountered several times by the Inspectorate for Health Protection and Veterinary Public Health (Keuringsdienst van Waren) and because several toy matrices with relatively high Pb levels were available. The three digestion models consist of three phases, mouth, stomach and small intestine, as mobilisation of Pb may occur in each phase. The three optimised digestion models were used to assess bioaccessibility of Pb from 4 toy matrices. All matrices were tested on two days for the suck and swallow model. Reproducibility of the in vitro digestions were determined by testing the bioaccessibility of all matrices on three to four days using the suck-swallow model. The applicability of the in vitro digestion models for exposure assessment of contaminants in relation to guidelines and CONSEXPO is discussed.

1.2 Framework of research on bioaccessibility of contaminants from toys

The research on bioaccessibility of contaminants from toys is performed in project V/320102 (formerly V/630030). The overall aim of the project is to develop and evaluate the use of *in vitro* methodologies in order to assess the internal exposure in man to ingested contaminants. Project V/32102 consists of two topics: research on the bioaccessibility of contaminants from 1) toys and 2) food. The present report describes the development and suitability of three in vitro digestion models for toys, i.e. the suck, suck-swallow, and swallow model, to assess bioaccessibility of Pb from several toy matrices. In the food part of the project an in vitro digestion model is developed that simulates the conditions in the gastrointestinal tract under fed conditions, whereas fasting conditions are simulated in the toy part of the project.

Project V/320102 is related to project I/320000/16/OB (formerly M/711701/01/OB). In project I/320000/16/OB an in vitro digestion model for contaminants ingested with soil has been developed, and experience with the model has been acquired during several years. This in vitro digestion model was the starting point for the development of the toy and food digestion models. In addition, both projects are related in the research on the validation of the models with Pb, because the first vitro - vivo validation is based on the bioavailability of Pb from soil under fed and fasted conditions. The vitro – vivo validation is further addressed in milestone “Haalbaarheidsstudie naar validatie van het in vitro digestiemodel” (delivered in 2001), milestone “Haalbaarheidsstudie naar combinatie in vitro digestiemodel met in vitro absorptie model” (delivered February 2003), and milestone “Validatie in vitro digestiemodel in vivo” (to be delivered in 2004).

The human exposure model CONSEXPO is developed in project V/320104 (formerly V/612810).

1.3 Outline of the report

In chapter 2 an introduction to internal exposure after ingestion (oral bioavailability) and bioaccessibility is given. The development of the three variants of the in vitro digestion model to simulate oral exposure to toys is described in detail in chapter 3. In chapter 4, bioaccessibility of Pb from several toy matrices is determined with the three variants of the optimised in vitro digestion model, and the results are discussed. The applicability of these models for exposure assessment of contaminants is discussed in chapter 5. The conclusions are briefly summarised in chapter 6.

2. Oral bioavailability and bioaccessibility of contaminants from toy matrices

2.1 Oral bioavailability: definition

The term oral bioavailability knows many interpretations, mostly depending on the field of research. In the present project we consider oral bioavailability of contaminants from toys to man.

Oral bioavailability is defined as the fraction of an external dose that results in internal exposure (see figure 1).
The *external dose* represents the total amount of a contaminant that a person is in direct contact with or ingests. The contaminant is considered "*internal*" if it is absorbed from the gastrointestinal tract, transported through the liver into the systemic circulation, i.e. the central bloodstream.
Oral bioavailability consists of three processes that are schematically presented in figure 1. First, the contaminant should be mobilised from its matrix into the juices of the gastrointestinal tract. This process is referred to as bioaccessibility. The mobilised contaminants can subsequently be transported across the intestinal epithelium into the portal vein. The fraction of the contaminant that passes the liver without being metabolised will reach the systemic circulation. Consequently, bioavailability (F) is the product of bioaccessibility (F_B), absorption (F_A), and metabolism (F_H), see figure 1.

2.2 Effect of matrix on bioavailability

The matrix in which a contaminant is present, for example, food, liquid, soil, toy, can affect the oral bioavailability of a contaminant. For Pb, it appeared that bioavailability from soil is considerable lower than bioavailability of lead salts in the diet (Dieter *et al.* 1993; Freeman *et al.* 1996; Freeman *et al.* 1992).

2.2.1 Effect of matrix on bioaccessibility

The matrix in which a contaminant is present plays an important role in bioaccessibility. The matrix affects the fraction of contaminant that is released into digestive fluid during sucking or during transit through the gastrointestinal tract after ingestion. Only the contaminant molecules that are released from the matrix in the small intestine are considered to be available for intestinal absorption.
Studies with an in vitro digestion model as developed earlier in laboratory have shown that a considerable fraction of contaminants remains associated with soil during digestion (Oomen *et al.* 2003; Oomen *et al.* 2002). Hence, the matrix of ingestion may lower the bioaccessible fraction, i.e. $F_B < 1$, and thus lower internal exposure.

2.2.2 Effect of matrix on absorption and metabolism

Compound specific properties, such as molecular weight, lipophilicity, affinity for P450 etc, determine the passage over the intestinal epithelium and the susceptibility for metabolism in the liver (Gan and Thakker 1997). As the matrix will not affect the compound specific properties, it is not expected that once released from the matrix, the matrix itself will have an effect on the absorption or metabolism of the contaminant. Nevertheless, in some cases the matrix of ingestion has been shown to affect the transport of the contaminant across the intestinal epithelium (Wienk *et al.* 1999). For example, food constituents may compete with the contaminant for transport across the intestinal epithelium. This is likely the case for minerals and metals. However, as transport across the intestinal epithelium and metabolism in the liver predominantly depend on compound specific properties, mostly it can be assumed that the matrix of ingestion does not affect the transport across the intestinal epithelium or the metabolism in the liver. In that case, the difference in bioaccessibility of a compound from two different matrices reflects the difference in bioavailability of the compound from the two different matrices.

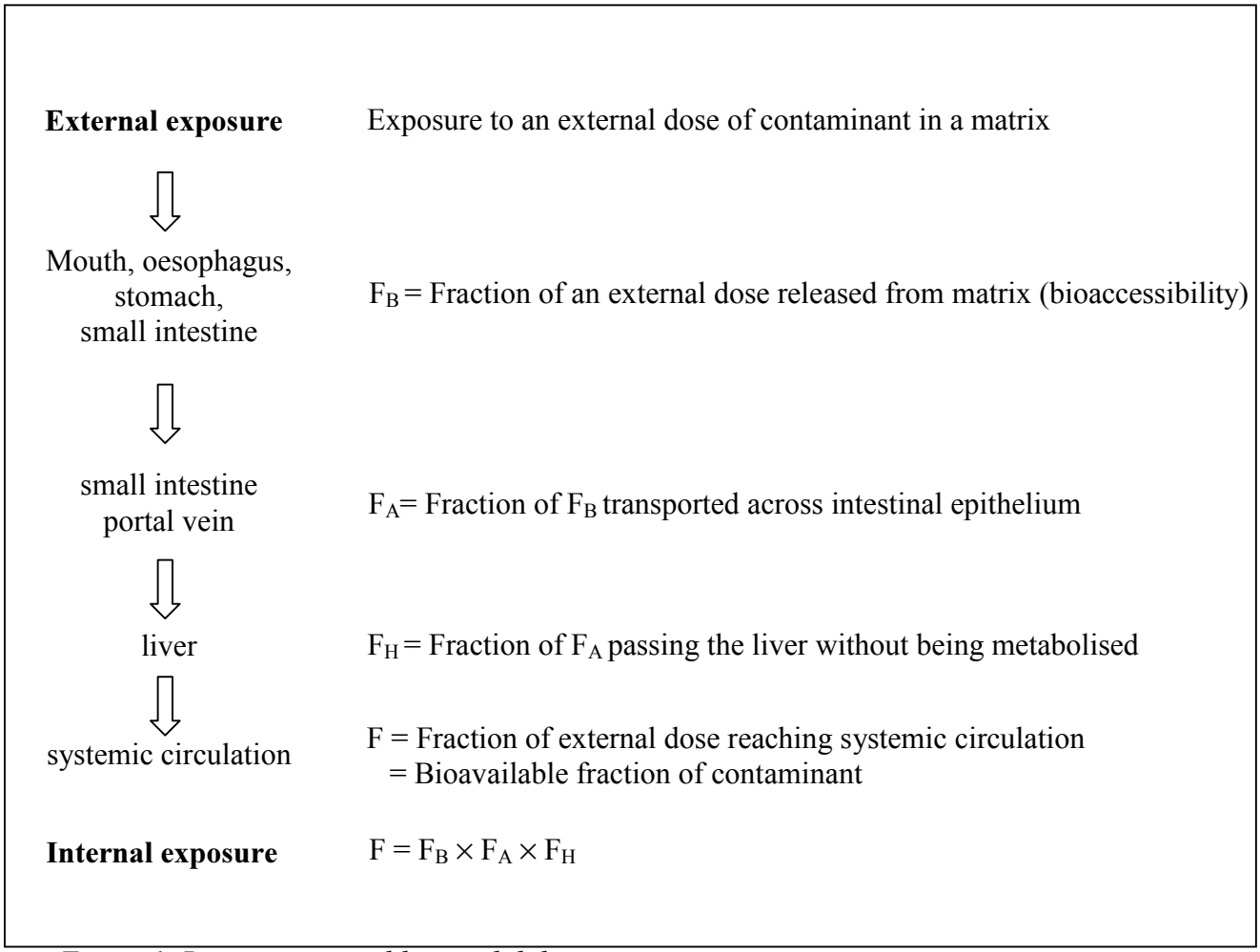

Figure 1. Processes in oral bioavailability.

2.3 Research on bioaccessibility

For contaminants in soil and in drug research, there is an increasing interest in the use of in vitro methodologies to study the human bioavailability of compounds. In this research, in vitro digestion models simulate the digestion process in the gastrointestinal tract in a simplified manner by applying physiological based conditions, i.e. chemical composition of digestive fluids, pH and residence time periods typical for each compartment. Most of the in vitro digestion models describe a two- (stomach and small intestine) or three-step procedure (mouth, stomach, small intestine or stomach, small and large intestine). The digestion procedure of a three-step procedure (mouth, stomach, small intestine) is schematically shown in figure 2. The bioaccessibility of the contaminant can be determined in each compartment. However, absorption of compounds takes mainly place in the small intestine and therefore, the bioaccessibility determined in the chyme of the small intestine is most relevant.
A similar approach as for contaminants in soil and in drug research can be applied for contaminants in toys. The in vitro digestion models described in the present report are developed for that aim.

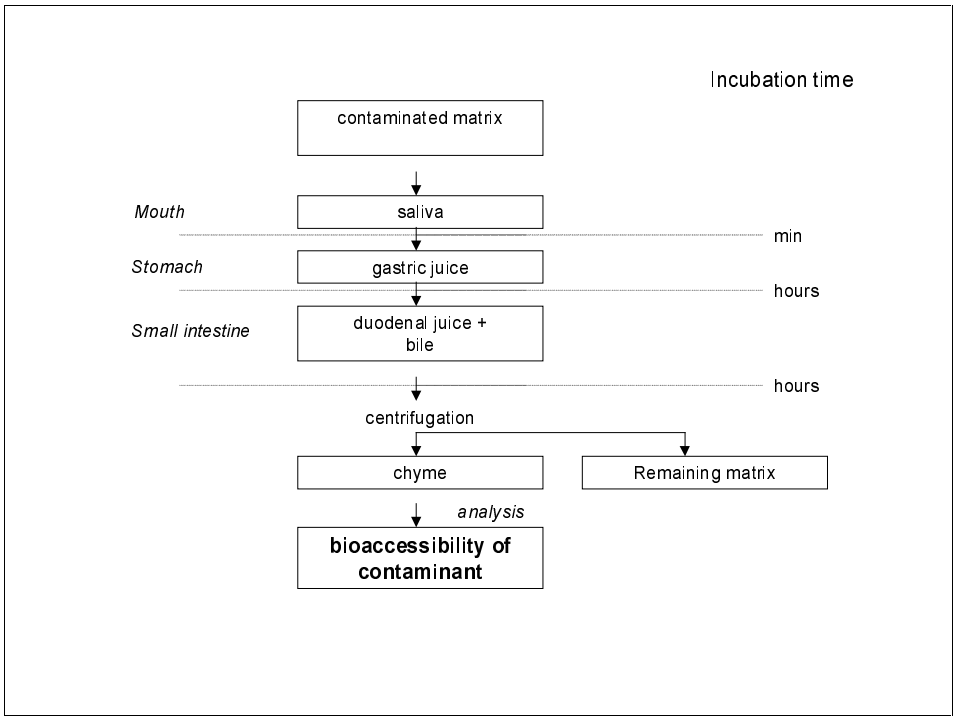

Figure 2. Schematic representation of an in vitro digestion model that follows a three-step procedure simulating the digestive processes in the mouth, stomach and small intestine.
In each compartment, the matrix is incubated at 37°C for a time relevant for the compartment. The digestion is initiated by addition of artificial saliva to the contaminated matrix. Subsequently, gastric juices and intestinal fluids are added to simulate the digestive processes in stomach and small intestine, respectively. Thereafter, the concentration of the contaminant in the chyme (intestinal content) is determined.

3. Development in vitro digestion models for toys

In the present chapter the development of three in vitro digestion models to assess the bioaccessibility of contaminants from toys is described. The following scenarios of handling toys are discriminated: 1) child sucks on a toy and swallows the contaminants released in saliva, 2) child sucks on a toy, swallows not only the contaminants released in saliva but swallows also the contaminated toy matrix, and 3) child swallows the contaminated toy matrix without a sucking phase. The in vitro models are referred to as 1) suck, 2) suck-swallow, and 3) swallow digestion model, respectively.

3.1 Starting point

3.1.1 Outline of the in vitro digestion models

As starting point for the design of the in vitro digestion models for contaminants in toys the digestion model for soil was used (Oomen *et al.* 2003). This consists of a three-step procedure that simulates the conditions in the mouth, stomach and small intestine (see figure 2).
A different ratio of digestive juices was proposed for the in vitro digestion models for toys than for the model for soil. The ratio for the soil model was 1 : 1.5 : 3 : 1 for saliva : gastric juice : duodenal juice : bile juice. This ratio of digestive juices was based on the volumes of juices that are produced in the human gastrointestinal tract (Guyton 1991; Tortora and Grabowski 1996). However, some of the juices are reabsorbed. Therefore, the ratio was adjusted to 1 : 2 : 2 : 1 for the toy models because this ratio closer represents the ratio as present in the human gastrointestinal tract, that is, accounting for the reabsorption of digestive juices during transit.

3.1.2 Composition of digestive fluids

Composition of the digestive fluids is based on human physiology as described in literature. As a starting point for the RIVM in vitro digestion models for soil and toy, the composition of artificial digestive fluids for a model described by Rotard *et al.* was used (Rotard *et al.* 1995), who in turn based theirs on Documenta Geigy (Ciba-Geigy, Basle, Switzerland). Some adaptations have been made from the Rotard model. Firstly, $NaHCO_3$ levels in duodenal juice were increased from 1.8 g/l to 3.4 g/l, and in bile, from 4.2 g/l to 5.8 g/l. These adaptations were necessary to obtain an instant pH change from the stomach (pH 1-1.5) to the intestine (pH>5.5) at the moment duodenal juice and bile are added to gastric juice. This change in pH also takes place in the human body. Secondly, higher bile levels were employed (0.9 g/l chyme versus 0.5 g/l chyme in the Rotard model) as these are closer to basal bile levels in humans (Charman *et al.* 1997; Hörter and Dressman 1997). Thirdly, different volumes of HCl were added to the different digestive juices so the pH values approximate the chosen ones.

The composition of the synthetic digestive juices (saliva, gastric juice, duodenal juice and bile juice) that are used in the in vitro digestion model for soil are described in detail by Oomen *et al.* (Oomen *et al.* 2003).

A different composition for stimulated (suck and suck-swallow model) and unstimulated (swallow model) was proposed on the basis of physiology. Sour taste, chewing, and smooth objects in the mouth stimulate the saliva production. The composition of the saliva is in turn dependent on the flow rate (Guyton 1991; Kedjarune *et al.* 1997; Salvolini *et al.* 1999). Therefore, the composition of stimulated saliva changed to higher levels of sodium, bicarbonate, and α-amylase, and a lower concentration of mucin than unstimulated saliva. Furthermore, the pH under stimulated conditions is slightly higher (Altman and Dittmer 1968; Guyton 1991; Kedjarune *et al.* 1997). The pH values of the digestive juices are presented in Table 1. The pH values are checked after preparation of the juices and, if necessary, adjusted to the appropriate interval with 1 M NaOH or concentrated HCl.

Table 1. pH values (± allowed interval) of the various synthetic juices of the suck, suck-swallow, and swallow in vitro digestion model.

Digestive juice	pH
Stimulated saliva	6.8 ± 0.2
Unstimulated saliva	6.5 ± 0.2
Gastric juice	1.40 ± 0.02
Duodenal juice	8.1 ± 0.2
Bile juice	8.0 ± 0.2

3.2 Development of the saliva phase

The three variants of the in vitro digestion model differ mainly from each other in the mouthing phase of the digestion process, i.e. sucking on a toy for a period of time or swallowing toy matrix. Chewing and smooth objects in the mouth stimulate the saliva production. Therefore, changes in the saliva phase are anticipated. These changes concern composition of the saliva, volume and duration of the saliva phase. In order to develop a physiological and practical saliva phase for each of the three models, experiments have been performed to study the effect of composition, volume and duration of the saliva phase on the bioaccessibility of Pb from finger-paint and chalk in saliva. Finger-paint and chalk were spiked at two concentrations, 0.25 and 1.5 mg Pb/gram matrix (chapter 4.1.2). These Pb concentrations were chosen because Pb concentrations of 0.25 mg/g and 2.0 mg/g had been found in a pilot experiment with paint chips and chalk powder, respectively (Van Veen 2001).

3.2.1 Composition of the saliva

In contrast to the composition of saliva in the experiments described in chapter 4, in the experiments described in this chapter 3.2, the mucin concentration was similar in the stimulated saliva and the unstimulated saliva.

To investigate effects of the saliva composition, finger-paint and chalk contaminated at two concentrations were incubated with 9 ml unstimulated and stimulated saliva for 30 min. Subsequently, the concentration of Pb in the saliva was measured (see table 2).
Bioaccessibility of Pb from finger-paint was 10-20% and was similar in stimulated and unstimulated saliva. Bioaccessibility of Pb from chalk was much lower (<1%). Almost all samples with stimulated saliva were below the minimal concentration of quantification suggesting that the release of Pb from chalk in stimulated saliva was slightly lower than in unstimulated saliva.

Table 2. Effect of composition of saliva on the bioaccessibility (%) of Pb from finger-paint and chalk in saliva. Data are means ± SD of 12 samples from 2 independent experiments.

	Pb Bioaccessibility in %			
	Finger-paint (0.25 mg/g)	Finger-paint (1.5 mg/g)	Chalk (0.25 mg/g)	Chalk (1.5 mg/g)
Unstimulated	9.8 ± 2.8	19.0 ± 4.9	<1.0 (6)*	0.36 ± 0.16
Stimulated	11.2 ± 2.0	19.8 ± 3.5	*	<0.09 (3)*

* In brackets the number of samples is given that were above the limit of quantification. The other samples were below the limit of quantification.

Although experimental data revealed that bioaccessibility was hardly affected by composition of saliva, it was decided to increase the pH and the concentrations of bicarbonate and amylase, and reduce the concentration of mucin compared to unstimulated conditions according to literature data.

3.2.2 Duration of the mouthing phase

In the swallow model the time a toy is in the saliva phase is very short. The duration may vary from several seconds to a few minutes. For practical reasons, 5 min was chosen in the development of in vitro digestion model for soil contaminants (Oomen *et al.* 2003).

A few references report the length of time that various toys are mouthed. The Wageningen Agriculture University conducted an observational study on the mouthing behaviour of children (see figure 3) (Groot *et al.* 1998). This study showed that duration of the saliva phase may vary from several minutes up to 90 minutes with a mean mouthing time of 26 min (SD 32). Similar mouthing times were observed by Juberg *et al.* of 28 min (SD 66) per day for children between 0 and 18 months (Juberg *et al.* 2001).

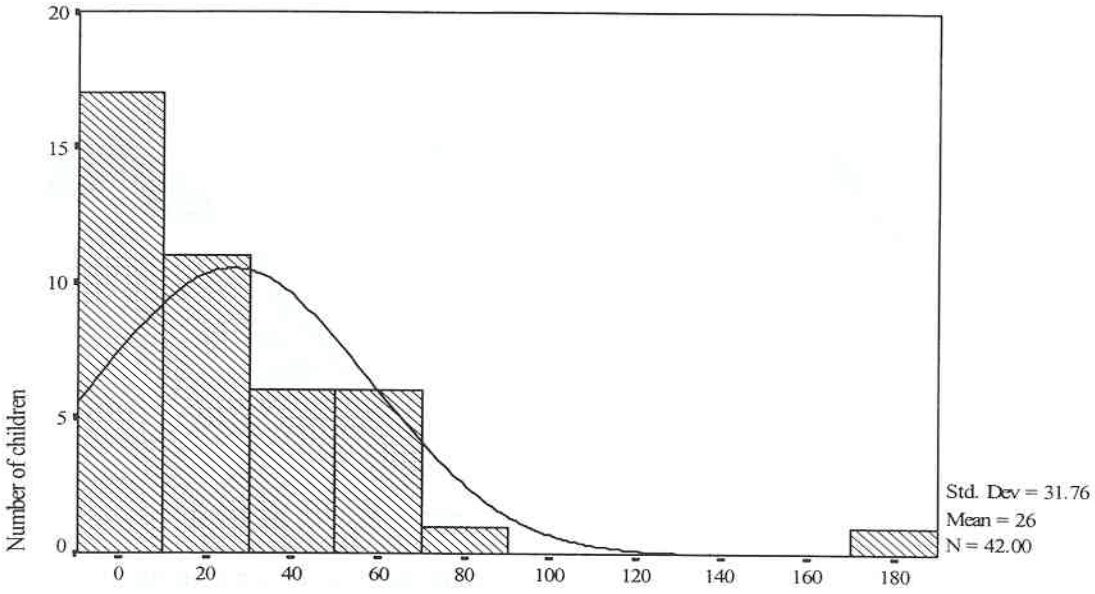

Figure 3. Mouthing times (excluding dummy) for children, 3-36 months, during day time.

To study the effect of mouthing time on bioaccessibility, bioaccessibility of Pb in saliva from finger-paint and chalk was determined as a function of time (5-120 min). Figure 4 shows that bioaccessibility of Pb from finger-paint increased during the first 15 minutes of incubation and remained constant up to 60 min. As thoroughly mixing of finger-paint with saliva took some time (>5 min), this is probably the reason for the increase in bioaccessibility of Pb from 5 to 15 min. For chalk no significant difference in bioaccessibility of Pb was observed at either time-point.

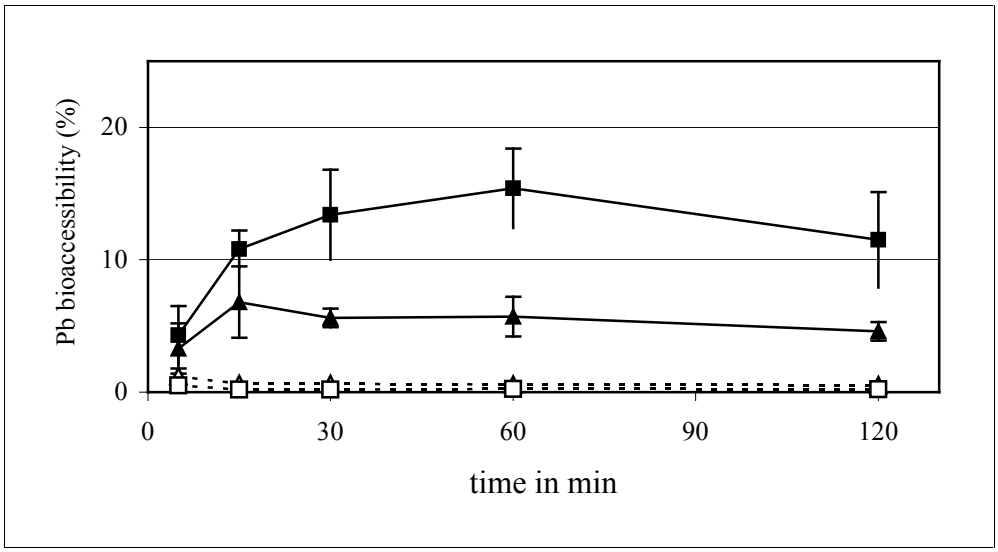

Figure 4. Bioaccessibility of Pb from finger-paint (closed symbols) and chalk (open symbols) in saliva as function of the incubation time.

The squares are the high contamination level (1.5 mg Pb/gram matrix) and the triangles are the low level of contamination (0.25 mg Pb/gram matrix). Data are the mean of 12 samples of two independent experiments.

No difference in bioaccessibility of Pb from finger-paint and chalk were measured between 15 and 60 min. As in observational studies described in literature mean mouthing times of 20-30 min were found (Groot *et al.* 1998; Juberg *et al.* 2001), 30 min incubation with the saliva was used to simulate the mouthing behaviour on toys in the suck and suck-swallow model. For the swallow model, 5 min incubation with the saliva phase was employed.

3.2.3 Saliva volume

The total amount of saliva produced will depend on the duration of sucking and on the saliva flow. Chewing and sucking on objects increases the saliva production on average 3 to 4-fold compared to rest condition (Foulkes and Bergman 1993; Fox *et al.* 1994; Guyton 1991; Navazesh *et al.* 1992). Increase of the saliva production automatically implies an increase in the frequency of swallowing. Because of practical reasons, only the effect of saliva volume and not the frequency of swallowing was studied (figure 5).

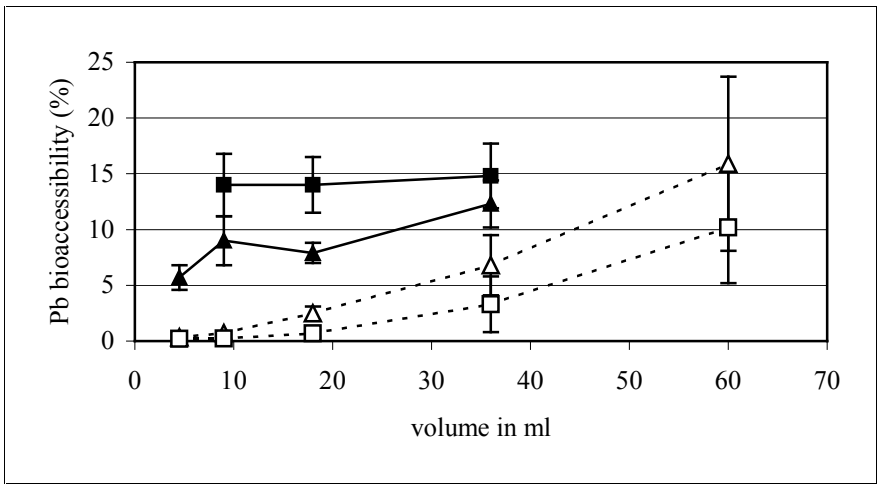

Figure 5. Bioaccessibility of Pb from finger-paint (closed symbols) and chalk (open symbols) in saliva as function of the saliva volume.
The squares are the high contamination level (1.5 mg Pb/gram matrix) and the triangles are the low level of contamination (0.25 mg Pb/gram matrix). Data are the mean of 12 samples of two independent experiments.

For chalk a clear correlation between saliva volume and bioaccessibility of Pb was observed. The bioaccessibility of Pb from the high contamination level of chalk increased from 0.2% at 4.5 and 9 ml saliva to 10% at 60 ml saliva. This suggests that the release of Pb from the chalk is concentration dependent probably caused by a solubility problem. On the other hand, no clear relation between the Pb bioaccessibility from finger-paint and the saliva volume was measured.

As sucking on objects stimulates the saliva production on average 3- to 4-fold, the volume of saliva in the suck and suck-swallow model was increased 3-fold. The experimental data showed that the saliva volume can be an important factor in the release of Pb from its matrix.

3.3 Design of the suck, suck-swallow and swallow model

3.3.1 General set-up digestion models

The general set-up of the digestion models is as follows and is schematically shown in figure 6. The digestion starts by introducing saliva to 0.4 g of toy (dry weight). This mixture is rotated head-over-heels for 5 min (swallow) or 30 min (suck and suck-swallow) at 55 rpm. In the suck model, the saliva is centrifuged during 5 min at 2750*g*, and 18 ml of the supernatant is transferred into a new tube. A sample of the remaining supernatant is taken to determine bioaccessibility in the mouth. To the tube with 18 ml saliva (suck) or the tubes with saliva and matrix (suck-swallow and swallow), 12 ml of gastric juice (pH 1.4 ± 0.02) is added, and the mixture is rotated for 1 h. The pH of the mixture of saliva and gastric juice is determined and subsequently set to pH as in the control tube without matrix. The pH in the gastric compartment of the swallow model is lower, pH 1.6, compared to the gastric pH in the suck and suck-swallow models, pH 2.1-2.5, because less saliva is entering the gastric compartment in the swallow model. The mixture is rotated for another h. Finally, 12 ml of duodenal juice (pH 8.1 ± 0.2) and 6 ml bile (pH 8.0 ± 0.2) are added simultaneously, and the mixture is rotated for another 2 h. The pH of the chyme is determined once more.

All digestive juices are heated to 37 ± 2 °C. Mixing takes place in a rotator that is also heated to 37 ± 2 °C. At the end of the in vitro digestion process, the digestion tubes are centrifuged for 5 min at 2750*g*, yielding the chyme (the supernatant), and the digested matrix (the pellet).

In chapter 3.1, further information on the composition and preparation of the digestive fluids is given.

3.3.2 Differences between digestion models

The main differences between the suck, suck-swallow, and swallow in vitro digestion model are addressed below.
Matrix. The suck model simulates the release of contaminants from toys during sucking. In contrast to the suck-swallow and swallow model, the matrix is not ingested. For that reason, the saliva + toy mixture was centrifuged at the end of the mouth compartment. The supernatant, i.e. the saliva without toy matrix, is used to continue the digestion process.
pH stomach. The matrix may affect the pH in the stomach. However, in fasting conditions, which are simulated in the digestion models, the pH in the stomach is usually low. For that reason, the pH in the stomach was set to 2.1-2.5 after 1 h in the suck and suck-swallow model. The pH of the stomach was set to pH 1.6 in the swallow model. The pH in the gastric compartment of the swallow model is lower compared to the gastric pH in the suck and suck-swallow models because less saliva is entering the gastric compartment in the swallow model. These pHs were chosen because an in vitro digestion without matrix results in this pH and because this pH falls in the range of pH values for fasting conditions (Charman *et al.* 1997).

Volume and composition of the saliva and the mouthing time. In the suck model, the volume is initially 21 ml, but 18 ml remains after removal of the matrix; the composition is of stimulated saliva, and the mouthing time is 30 min. For the suck-swallow model 18 ml stimulated saliva and mouthing time 30 min are employed. The swallow model simulates direct ingestion of a toy. Therefore, 6 ml unstimulated saliva and incubation of 5 min are employed for the swallow model.

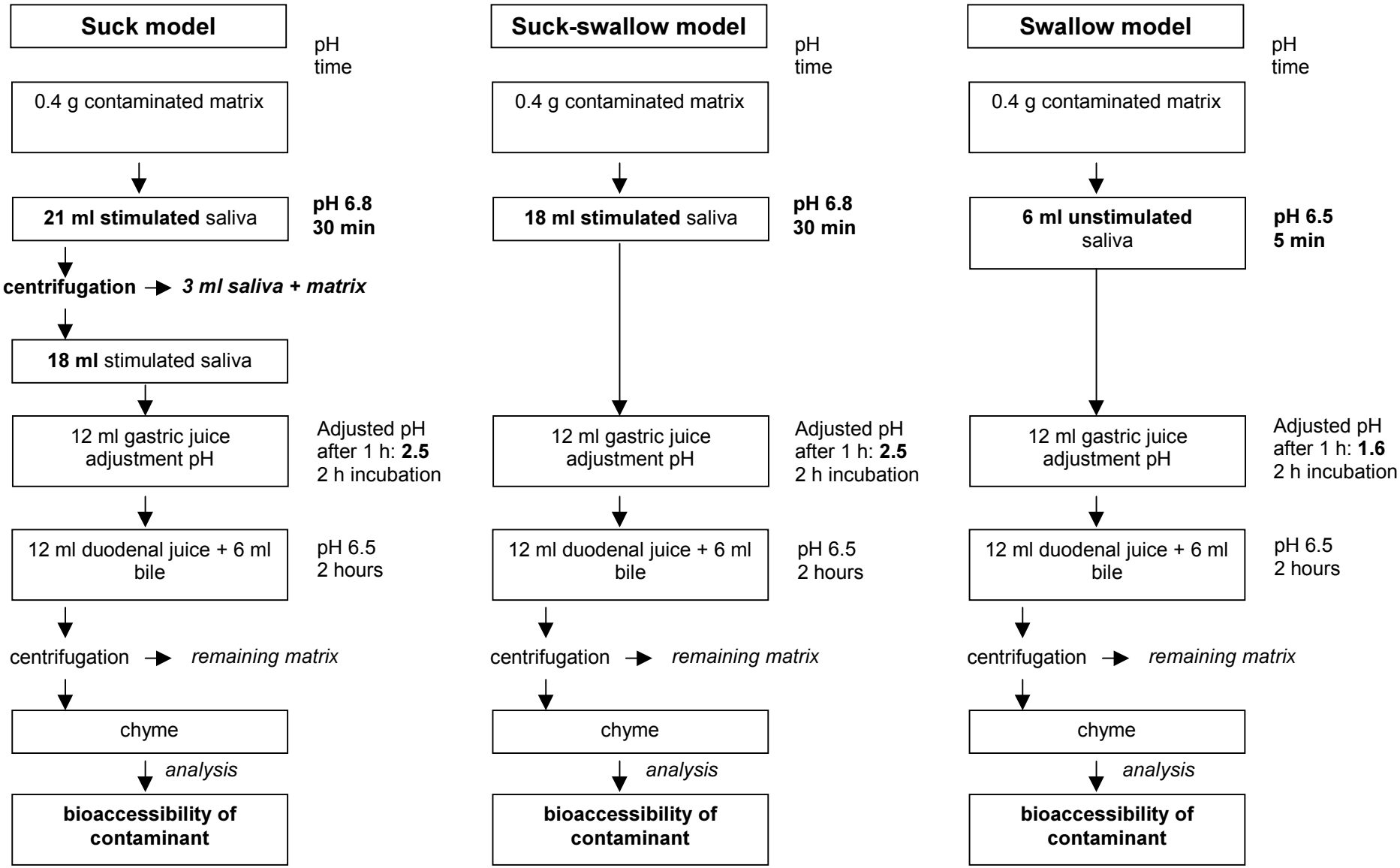

Figure 6. Schematic representation of the suck, suck-swallow and swallow in vitro digestion models. Differences between the models are rendered in bold.

4. Bioaccessibility testing

The present chapter describes some experiments on bioaccessibility testing of Pb in toy matrices, using the optimised in vitro digestion models for toys, i.e. the suck, suck-swallow and swallow model.

4.1 Materials and Methods

4.1.1 Test compound

Bioaccessibility was investigated for the contaminant Pb in toy matrices. The reasons for choosing Pb as a contaminant were mainly practical. The analysis of Pb in chyme and the method of destruction of pellet samples were available and several matrices with Pb were available. In addition, the issue of Pb in toy matrices has been encountered several times by the Inspectorate for Health Protection and Veterinary Public Health (Keuringsdienst van Waren).

4.1.2 Matrices

The following matrices contaminated with Pb were used in the digestion experiments: chalk, chalk for exterior use (stoepkrijt), finger-paint, SRM 2581 paint and Montana Soil 2711. The Pb concentrations in the matrices varied from 0.25 mg/g matrix to 28 mg/g matrix (see table 3). The Pb concentrations were either certified by the manufacturer (SRM 2581 paint and Montana Soil 2711) or were spiked in the laboratory (chalk and finger-paint) and/or determined experimentally by destruction of the matrices (chalk for exterior use).

Table 3. Concentration of Pb in the matrices.

Matrix	Concentration Pb (mg/g matrix)	Manner of contamination
Chalk	1.5	Spiked
Chalk for exterior use	28	Contaminated in production process
Finger-paint	0.25	Spiked
SRM 2581 paint	4.49	Contaminated in production process – certified
Montana Soil 2711	1.16	Not relevant (no toy matrix) – certified

Preparation of spiked matrices
Chalk was contaminated with Pb in the laboratory. To that end, $Pb(NO_3)_2$ was ground fine in a mortar. Subsequently, 600 mg $Pb(NO_3)_2$ were added to 250 g chalk, and mixed extensively by mechanical rotation and by hand.

Finger-paint was contaminated with Pb in the laboratory. To that end, 178 mg $Pb(NO_3)_2$ was dissolved in 2.0 ml milli-Q. Of this solution 1.8 ml was added to 400 g finger-paint. Finger-paint was mixed extensively by mechanical rotation and by hand. The concentration of Pb in finger-paint is thus 0.25 mg/g wet weight.

The liquid content of each matrix was determined by drying the matrices at a temperature of 105 ºC till there was no more change in weight. Finger-paint consists for 50% of water and powdered chalk for 0.7%.

The Pb concentration in chalk and finger-paint were experimentally confirmed by destruction experiments.

Historically contaminated matrices
Paint contaminated with Pb was obtained from the NIST (National Institute of Standards & Technology, US), and referred to as SRM (Standard Reference Material) 2581. SRM 2581 is composed of paint collected from the interior surfaces of housing. The paint flakes were smaller than 100 μm in diameter.

Chalk for exterior use was obtained from the Inspectorate for Health Protection and Veterinary Public Health (Keuringsdienst van Waren) without further specifications. The chalk was highly contaminated with Pb as shown in table 3.

Montana 2711 soil is a standard reference soil prepared by NIST. This soil material was dried in an air-drying oven at room temperature, sieved through a 2-mm screen and subsequently crushed to pass through a 74-μm sieve. The bioaccessibility of Pb from Montana 2711 soil has also assessed by the in vitro digestion model for soil and an in vitro digestion model that simulates fed conditions.

4.1.3 Experimental set-up

All matrices were digested in the suck, suck-swallow, and swallow in vitro digestion model on respectively 2, 3-4, and 2 separate days. At least three replicates, i.e. digestions in separate tubes with the same matrix, were used within a digestion experiment, i.e. one model on one day.

4.2 Results

4.2.1 Suck model

pH in stomach and intestinal compartment
The pH of the mixture of saliva and gastric juice in the stomach compartment and the pH of the chyme in the intestinal compartment were similar to the pHs in the test tube without

matrix. The pH was 2.5 ± 0.1 (N=42) in the stomach and 6.6 ± 0.2 (N=42) in the intestinal compartment.

Bioaccessibility in mouth, stomach and intestinal compartment
Concentration of Pb was measured in saliva (mouth), in the stomach compartment, and in the intestinal compartment. The results in table 4 are the mean of 2 independent experiments and expressed as % bioaccessibility.

Table 4. Bioaccessibility of Pb in mouth, stomach and intestine in suck model.

Matrix	N	Bioaccessibility of Pb in %		
		Mouth	Stomach	Intestine
SRM paint	6	0.4 ± 0.2	BLQ	0.3 ± 0.1
Chalk for exterior use	6	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
Chalk	12	BLQ	BLQ	BLQ
Finger-paint	12	8.4 ± 1.5	BLQ	5.4 ± 0.9
Montana Soil	6	13 ± 13	BLQ	<1*

BLQ = below limit of quantification. Limit of quantification were for chalk 0.3% in mouth, 4.0% in stomach, and 0.6% in intestine; 1.3% for SRM paint, 0.2% for chalk exterior, 24% for finger-paint, and 5.2% for Montana soil in stomach, respectively.
* Only 1 out of 6 samples was above the limit of quantification = 0.8%.

Release of Pb from its matrix into saliva was in general low. The bioaccessibility of Pb from chalk, chalk for exterior use and SRM paint in saliva was even less than 1%. The bioaccessibility from finger-paint and Montana Soil was approximately 10%. The variation in bioaccessibility of Pb was low from all matrices except from Montana Soil. We have found no technical, experimental reasons for this.

The concentration of Pb in the stomach could not be determined adequately in most cases. Because of technical problems with the detection of Pb in gastric juice, the samples had to be diluted 100-fold for analysis. This resulted in concentrations of Pb below the limit of quantification.

In the intestinal compartment, the concentration of Pb could be determined reliable for SRM paint, chalk for exterior use and for finger-paint. For these three matrices the bioaccessibility of Pb was comparable to the bioaccessibility in the mouth ("bioaccessibility intestine/bioaccessibility mouth" was 1.1, 1.4 and 0.64 for SRM paint, chalk for exterior use and finger-paint, respectively). As for chalk the concentration of Pb was already too low to determine in the saliva, no Pb could be determined in the chyme. Only in 1 out of 6 samples of Montana Soil, Pb could be determined in the chyme. Apparently, something was released from Montana Soil in saliva what affected the bioaccessibility of Pb further on in the gastrointestinal tract.

4.2.2 Suck-swallow model

pH in stomach and intestinal compartments
Gastric juice was added to the matrix after 30 min incubation with saliva. The pH was measured after 1h incubation in the stomach compartment. The matrices used in this study highly increased the pH in the stomach compartment. Chalk for exterior use increased the pH in the stomach to 3.5 whereas the other 4 matrices increased to pH 5. Although this is a physiological stomach pH after eating a meal, the physiological stomach pH for fasted conditions is ~2 (Charman *et al.* 1997). Therefore, the pH in the stomach compartment was adjusted after 1 h incubation in the stomach compartment, by addition of concentrated HCl, to the pH in the stomach compartment without a matrix present (pH 2.5). Thereafter, the matrix was incubated in the stomach compartment for another hour and the pH was measured afterwards. For Montana Soil and chalk the pH was similar at adjustment and after 1h incubation. However, the pH in the stomach compartment was increased after 1h incubation for SRM paint (pH 3.5), finger-paint (pH 5.2) and chalk for exterior use (pH 3.2).
The variation in pH in the intestinal compartment was much smaller: the lowest pH was 6.0 for chalk and the highest pH was 6.9 for finger-paint.

Bioaccessibility of Pb in stomach and intestinal compartments
The concentration of Pb was measured in the stomach compartment and in the intestinal compartment. The results in table 5 are mean of 3 to 4 independent experiments and expressed as % bioaccessibility.

Table 5. Bioaccessibility of Pb in stomach and intestine in suck-swallow model.

Matrix	N	Pb Bioaccessibility in %	
		Stomach	Intestine
SRM paint	9	22 ± 6	10 ± 6
Chalk for exterior use	9	50 ± 11	0.1 ± 0.0
Chalk	24	57 ± 18	0.8 ± 2.2
Finger-paint	18	55 ± 11	17 ± 2
Montana Soil	12	64 ± 22	23 ± 5

The bioaccessibility of Pb was higher in the stomach than in the intestinal compartment for all matrices. Because of the low pH in the stomach this was an observation to be expected. Independent of the matrix, the bioaccessibility of Pb in the stomach was ~50%-60%, except for SRM paint for which the bioaccessibility was only ~20% in the stomach.

In contrast to bioaccessibility of Pb in the stomach, which was comparable for most matrices, bioaccessibility in the intestinal compartment varied strongly per matrix. The bioaccessibility of Pb was more than 50-fold lower in the intestinal compartment compared to the bioaccessibility in the stomach for both chalk matrices. Bioaccessibility of Pb decreased only 2 to 3-fold for the other three matrices. Thus, the amount of Pb released from its matrix in the stomach is not predictive for the amount of Pb available for absorption in the intestine, but is very much dependent on the matrix.

4.2.3 Swallow model

pH in stomach and intestinal compartments
The main difference between the suck-swallow and the swallow model is that 3-fold less saliva is used in the mouth compartment. This results in less dilution of the gastric juices in the stomach and, therefore, the pH in the stomach is lower in the stomach of the swallow model (pH 1.6) than in the suck-swallow model (intended pH of 2.3-2.5). As the matrices increased the pH in the stomach compartment to pHs higher than pH 5 (see above suck-swallow model), the pH in the stomach for each matrix was adjusted by addition of concentrated HCl to the stomach pH without matrix present, pH 1.6. Only for finger-paint, the pH in the stomach sometimes increased after incubation for another hour, the pH in the stomach varied between pH 1.5 and pH 4.1.
The pH in the intestinal compartment pH 6.4 was comparable with the intestinal pH in the suck-swallow model.

Bioaccessibility of Pb in stomach and intestinal compartments
Similar to the suck-swallow model, bioaccessibility of Pb in the stomach was higher than in the intestine (table 6). Bioaccessibility of Pb in the stomach in the swallow model was even higher than in the suck-swallow model, 52-92% bioaccessibility compared to 22-64%, respectively, which can be explained by the lower pH of the stomach in the swallow model.

Also similar to the suck-swallow model was that the bioaccessibility of Pb in the intestinal compartment was very dependent on the matrix, with bioaccessibility between 1.6% and 51%. Bioaccessibility of Pb in the intestinal compartment in the swallow model was in general higher compared to the bioaccessibility in the suck-swallow model, again with the lowest bioaccessibility of Pb <5% for both chalk matrices. Bioaccessibility of Pb in the intestinal compartment was moderate for both paint matrices, 25% and 51 % for SRM paint and finger-paint, respectively.

Table 6. Bioaccessibility of Pb in stomach and intestine in swallow model.

Matrix	N	Pb Bioaccessibility in %	
		Stomach*	Intestine
SRM paint	6	52 ± 1	25 ± 4
Chalk for exterior use[#]	3	59 ± 8	3.3 ± 0.4
Chalk	12	88 ±14	1.6 ± 1.1
Finger-paint	12	92 ± 3	51 ± 11
Montana Soil	6	86 ± 9	9 ± 5

* Bioaccessibility of Pb in stomach was determined in one experiment, whereas the bioaccessibility in intestine was determined in 2 experiments.
[#] The bioaccessibility of Pb from chalk for exterior use was only determined in 1 experiment in triplicate.

4.2.4 Methodological parameters

Mass balance
As a control for the method, mass balance of Pb was determined for all three models by measurement of Pb concentration in saliva (suck model) or chyme (suck-swallow and swallow model) and in the corresponding pellets (see figure 6). Mass balance was in general good with a mean recovery of 91%.

Reproducibility
Reproducibility within and between days was calculated for all 5 matrices based on the bioaccessibility in the stomach as well as the intestinal compartment of the suck-swallow model, because this model was repeated for 3 to 4 days (see table 7). The suck and swallow models were only repeated twice. Additionally, reproducibility was calculated for finger-paint and chalk based on the bioaccessibility in saliva of the experiments as described in chapter 3.2.

The within day variation was highest for chalk in the intestinal compartment. The low absolute values of the mean bioaccessibility and its standard deviation result in high within and between day variation. In most other cases the within day variation was <25%, which is acceptable for such a complicated method.
The between day variation was higher than the within day variation in all compartments.

There was no relation between the contamination level and between day variation or between matrix and between day variation on the other hand. The within day variation and between day variation of Montana soil were comparable with previous data for bioaccessibility of Pb (wdv 18 % and bdv 26 %) in the in vitro digestion model for soil contaminants (Zeilmaker *et al.* In preparation).

Between-day variation was high. This may be caused by the pH adjustments in the stomach, which had not yet been standardised in these experiments. For example, the bioaccessibility of Pb from SRM paint was in one experiment much lower than in the other 2 experiments, 3.1% vs 15.2% and 12.6%, respectively. This might be due to the higher pH in the stomach pH 3.8 vs pH 3.2 in the first experiment. Therefore, to reduce the between day variation it is advised to further standardise the preparation of the digestive fluids and especially standardise of the adjustment of pH in the stomach.

Table 7. Reproducibility of Pb bioaccessibility in saliva, gastric content and in chyme as determined by within (wdv) and between days (bdv) of the suck-swallow model.

	Saliva			Gastric content			Chyme		
Matrix	Mean ± SD	Wdv %	Bdv %	Mean ± SD	Wdv %	Bdv %	Mean ± SD	Wdv %	Bdv %
SRM paint				22 ± 6	11	53	10 ± 6	17	109
Chalk for exterior use				50 ± 11	17	30	0.1 ± 0.0	33	29
Chalk	0.2 ± 0.1	29	73	57 ± 8	25	61	0.8 ± 2.2	270	227
Finger-paint	8 ± 3	21	73	55 ± 11	9	53	17 ± 2	5	25
Montana Soil				64 ± 22	13	63	23 ± 5	16	30

* The number of replicates per experiment was N=6 for chalk and finger-paint, and N=3 for chalk for exterior use, SRM 2581 paint and Montana Soil 2711. Number of experiment was 3 or 4. The reproducibility of the Pb bioaccessibility in saliva was calculated on the experiments (9 ml stimulated saliva, 30 min incubation, N=6 replicates, 6 days) described in chapter 3.2. The matrices were finger-paint and chalk.

4.3 Discussion

4.3.1 Differences between suck, suck-swallow and swallow in vitro digestion models

In figure 7, a comparison between the three models has been made on basis of the bioaccessibility of Pb in chyme in the small intestinal compartment. Bioaccessibility was lowest for the suck model. This is not surprising because the conditions in the mouth are not very stringent for Pb; that is, the saliva is not very acid and does not contain many complexing agents for Pb. In the suck model the matrix is removed after the mouthing phase so that no further Pb can be mobilised from the matrix during digestion in the stomach and intestine. It appeared that for the toy matrices approximately all Pb that was bioaccessible in the mouth, remained bioaccessible in the intestinal compartment (table 4).

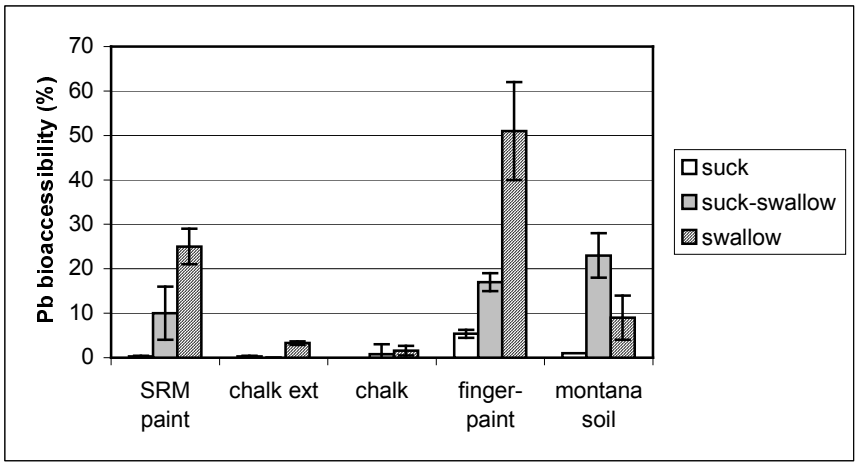

Figure 7. Comparison of suck, suck-swallow and swallow model determined by the bioaccessibility of Pb in chyme.

The toy matrix is ingested in the suck-swallow and in the swallow digestion models. Because the matrix is subjected to the acid environment in the stomach and the complex intestinal fluid, more Pb is bioaccessible in the stomach and intestine compared to the suck model. The acid environment in the stomach is very important for the release of Pb from the matrix. Because the gastric fluid is diluted with less saliva in the swallow model (see figure 6), the pH in the stomach of the swallow model was lower (pH 1.6) compared to the (intended) pH 2-2.5 in the suck-swallow model. This resulted in a higher bioaccessibility of Pb in the stomach in the swallow model (50-90%) than in the suck-swallow model (20-60%). The higher bioaccessibility in the stomach in the swallow model resulted also in a higher bioaccessibility of Pb in the small intestine in the swallow (1.6-51%) compared to the suck-swallow (0.1-23%) model (except for Montana soil).

In conclusion, the results are consistent with the different scenarios for oral exposure. The toy matrix is not ingested in the suck model and consequently Pb can only be released in the saliva, whereas Pb is mobilised from the toy matrix in the stomach and small intestinal compartment in the suck-swallow and swallow model.

4.3.2 Effect of the toy matrix on the bioaccessibility of Pb

Effects of the matrix on bioaccessibility of Pb have been demonstrated convincingly in all three models. Bioaccessibility of Pb in saliva varied from <0.2% to 13% with the lowest bioaccessibilities for both chalk matrices. The mobilisation of Pb from chalk in saliva but not from finger-paint was strongly dependent on the saliva production (figure 5). Finger-paint and chalk were spiked with Pb as $Pb(NO_3)_2$ at the same concentration. The difference in bioaccessibility and behaviour illustrates the importance of the matrix for the bioaccessibility, and thus oral bioavailability since the initial speciation of Pb and the contamination levels were the same in both matrices.

In contrast to bioaccessibility of Pb in saliva, bioaccessibility of Pb in the stomach was comparable for most matrices. The high bioaccessibility of Pb in the stomach, 50-90% in the swallow model, showed that Pb could be mobilised from its matrix.

The bioaccessibility of Pb in the small intestinal compartment was again very dependent on the matrix. The bioaccessibility of Pb was 20- to 50-fold lower in the intestinal compartment compared to the bioaccessibility in the stomach for both chalk matrices, whereas the bioaccessibility of Pb decreased only 2- to 3-fold for both paint matrices.

In conclusion, the data described in this report illustrate convincingly that bioaccessibility, and thus oral bioavailability, of Pb is strongly dependent on the matrix with which it is ingested.

4.3.3 Study parameters

Factors likely to affect the release of a compound from its toy matrix besides characteristics of the matrix and the compound themselves are the contamination level of the matrix, the origin (speciation) of contamination, the time the toy matrix is in contact with the gastrointestinal fluids, the dilution of the matrix by the gastrointestinal fluids, the amount of matrix ingested, and the physiological state of the gastrointestinal tract.

Level of contamination
The level of contamination is one of the factors potentially affecting bioaccessibility of a compound. In general, a linear (dose proportional) relationship between contamination level and bioaccessibility c.q. bioavailability is assumed. The assumption simplifies risk assessment, since regardless of the level of contamination, a constant percentage of the contaminant will be bioaccessible c.q. bioavailable. For artificially contaminated soils the relationship between contamination level and amount of Pb mobilised from the soil was linear in the range from 0.25 mg/g to 2.5 mg Pb/g soil (Zeilmaker *et al.* In preparation). Bioaccessibility of Pb from soil was dependent on the soil type but constant with the contamination level.

The data with $Pb(NO_3)_2$ contaminated finger-paint and chalk showed that the contamination level was a far less important factor for bioaccessibility of Pb in saliva than the matrix itself (chapter 3.2). The concentrations of Pb in the 4 toy matrices used varied a 100-fold from 0.25 mg/g finger-paint to 28 mg/g chalk for exterior use (table 3). The latter concentration is extremely high and it is likely that the amount Pb is exceeding the solubility and thus affecting the bioaccessibility of Pb. However, bioaccessibility of Pb from chalk with a much lower contamination level 1.5 mg/g chalk was also very low. The amount of Pb released from this matrix was the lowest of all matrices, whereas the contamination level was the median of the 5 matrices used.

Artificially contaminated versus production process contaminated matrices
Finger-paint and chalk were spiked in the laboratory whereas SRM-paint and chalk for exterior use were contaminated with Pb during the production process. As bioaccessibility of Pb was the highest from finger-paint and low(est) from chalk, the way the matrix was contaminated with Pb was not a determinant for bioaccessibility of Pb.

Time dependent release in relation to the matrix
Our data show that bioaccessibility of Pb from a toy matrix depends on the way of mouthing (sucking, swallowing) and on the matrix itself. In general, the shape and thickness of the product will be important, as only the outside of the product is in direct contact with the gastrointestinal fluids and only compounds that are located at the outside (surface area) of the product can be released into the fluids. The compound that is more located inside the product first has to migrate to the surface area of the product before it can be released. The rate at which compounds are released from their matrix is then often a diffusion-limited process. This is characterised by a time dependent release of the compound from its matrix until equilibrium has been reached. Such as time dependent release was shown for the release of the plasticizer DINP from PVC products (Könemann 1998).

In the study in this report, all matrices were homogenised into very small pieces (chalk 2x, SRM paint, Montana soil) or were on fluid basis (finger-paint). Therefore, the contact surface area of all matrices with the gastrointestinal fluids was large and Pb in the matrix was in "direct" contact with the gastrointestinal fluids. Hence, the release of Pb from its matrix is not to be expected diffusion limited. Indeed there was no correlation between the incubation time and the bioaccessibility of Pb in saliva other than the time it took to mix finger-paint thoroughly with the saliva (figure 4).

Amount of matrix ingested and dilution by gastrointestinal fluids
A child can directly put a toy in his mouth (object-to-mouth bahaviour), but also indirect contact via hand-to-mouth behaviour is possible. In general, the amount ingested via hand-to-mouth behaviour will be much lower than via object-to-mouth bahaviour. It has not been investigated in this study how bioaccessibility of Pb was related to the amount of toy ingested. Figure 5 shows that the bioaccessibility of Pb from chalk was highly dependent on the volume of saliva used. On the other hand the bioaccessibility of Pb from finger-paint was independent on saliva volume. As the ratio "amount toy matrix ingested / volume saliva" can be changed similarly by either using different volumes of saliva (as was done in figure 5) or by ingestion of a different amount of toy matrix, it is likely that the amount of toy matrix ingested can have an effect on the bioaccessibility of Pb.

The difference between object-to-mouth and hand-to-mouth behaviour will be investigated in future studies with SRM paint and chalk for exterior use by studying the effect of the amount of matrix ingested on the bioaccessibility of Pb.

Physiological state
The digestion models described in this report are based on the physiology of man under fasted conditions. In response to eating food not only the production of saliva increases but also the production of gastric juices and small intestinal juices increases. As a result, a toy

will be more diluted with the gastrointestinal fluids under fed conditions than under fasted conditions. Not only the production of gastrointestinal juices is stimulated under fed conditions but also the composition of the fluids and the physiological conditions in the stomach and small intestine. For example, under fasted conditions the pH in the stomach is lower than under fed conditions. The low pH in the stomach is very important for the release of Pb from its matrix (Oomen *et al.* 2002). This was also apparent from the difference in bioaccessibility between the suck-swallow and swallow model described in this report and tables 4 and 5. On the other hand, under fed conditions more complexing agents are present in the small intestinal fluid, which might improve the bioaccessibility of Pb (and other contaminants) (Oomen 2000; Oomen *et al.* 2002). Further research within this project will provide information on this topic (Versantvoort *et al.* In preparation).

4.3.4 Applicability of digestion models

Reproducibility – recommendations for future studies
Despite the high between-day variation the models are useful to estimate the bioaccessibility of Pb from a certain matrix (mean ± SD) in terms of very low (e.g. <10%), low (e.g. 10-30%), moderate (e.g. 30%-70%) and high (e.g. >70%). Differences between matrices or other variables observed in one experiment are in general consistently found when repeated although the absolute figure may differ. It is recommended to have the variables you want to compare within one experiment rather than spread the variables over different days. It is not necessary to have many replicates in one experiment because the within-day variation was less than 25% except for the very low bioaccessibilities. When it is acceptable to classify the bioaccessibility into different scales or comparison of the bioaccessibility of a compound from different matrices within the same experiment, one can sustain with 1 or 2 experiments with 3 to 4 replicates, including a relevant reference matrix. The more an experiment is repeated the more reliable the absolute bioaccessibility will be.
In order to allow for diffusion out of the toy matrix and because mouthing time is also highly variable amongst children, it is recommended to use at least two different incubation times to determine the bioaccessibility of compounds from a toy matrix in saliva.

Use of reference material
In future experiments different matrices will be used from series to series. To be able to assess whether the in vitro digestion has succeeded, a comparison between series is desirable. To that end, a reference sample should be included in every series. In the present experiment the bioaccessibility of Pb from two candidate reference samples has been determined, i.e. SRM 2581 paint and Montana soil 2711. Both matrices can be obtained commercially and are certified to be constant in time, homogeneous, and contain a certified Pb concentration.

The bioaccessibility of Pb from Montana soil 2711 has also been assessed by the in vitro digestion model for soil contaminants and an in vitro digestion model that simulates fed conditions. The within-day variation and between-day variation of the bioaccessibility of Pb from Montana soil 2711 in the suck-swallow model (wdv 16% and bdv 30%) were comparable with previous data (wdv 18 % and bdv 26 %) in the in vitro digestion model for

soil contaminants (Zeilmaker *et al.* In preparation), and with the variations found in the in vitro digestion model that simulates fed conditions (13% and 37% for the first infant formula and 15% and 11% for the second infant formula) (Versantvoort *et al.* In preparation). Thus, the data with Montana soil 2711 seem to be reproducible and consistent with different digestion models. However, it should be noted that the standard deviations for the suck and for the swallow model were rather high. For SRM 2581 paint the between-day variation was much higher (109%) probably due to a difference in stomach pH between the experiments. Although it is preferable to have a relevant matrix as a reference material, at this moment most consistent data are obtained with Montana soil 2711. Therefore, Montana soil 2711 should be included in future experiments as a reference material.

5. Application of the in vitro digestion models in risk assessment

Guidelines to determine safety of toys contaminated with heavy metals are based on migration of the heavy metals from the toy under acid conditions, i.e. HCl of pH 1-1.5 (European Toy Directive 88/378/EEC, NEN-EN 71-3). The conditions in the gastric compartment of our digestion models are comparable, although the pH is slightly higher (pH 1.6-2.5) and composition of the gastric content is more complex. Absorption of Pb and other compounds mainly occurs in the small intestinal compartment. Therefore, bioaccessibility in the small intestinal compartment is more representative for internal exposure than the bioaccessibility in the stomach. The bioaccessibility of Pb was higher in the stomach than in the small intestinal compartment for all matrices. Therefore, a risk assessment based on the migration of Pb from a toy under acid conditions (≈ bioaccessibility in the stomach) will overestimate the internal exposure and will overestimate the actual risk. The data in this report show that the difference between the bioaccessibility of Pb in the small intestine and stomach was very much dependent on the matrix: for the two chalk matrices the bioaccessibility in the small intestine was 20-to 50-fold lower compared to the bioaccessibility in the stomach but only 2- to 3-fold lower for the two paint matrices. Thus, the migration of Pb from a toy matrix determined under acid conditions will give a fast but a worst case indication for the internal exposure of the child to Pb. The in vitro digestion models can be useful when a more thorough risk assessment is needed.

In a study by CEN, it appeared that for several chemicals in a PVC disk (acetyltributyl citrate, phenol, naphthalene, isophorone, phenol, tricresylphosphate, xylene, benzylalcohol) water could be used instead of saliva simulant for mobilisation of the chemicals, although reproducibility between laboratories was in some cases bad (Strikwerda 2002; Hillersborg 2002). In future studies with the in vitro digestion models described in the present manuscript, both water and saliva will be used for comparison.

For plasticizers and other compounds in teethers and other toys, that are likely to be mouthed but not swallowed, the guidelines are not based on the migration of the compound under acid conditions but based on the migration in artificial saliva (Könemann 1998). It is assumed that the amount of compound that is released in saliva becomes bioavailable. Our conditions in the mouthing phase of the suck model should be comparable with the migration in artificial saliva. Future experiments with DINP from PVC disks, which were used in a validation study and interlaboratory study (Könemann 1998) will be used to compare the mouthing phase of the suck model. By also performing the second (stomach) and third step (small intestine) of the in vitro digestion model, more information is gained on the behaviour of the compound at the absorption site.
It should be kept in mind that the bioaccessibility of a compound in the intestinal compartment can be greater when a small amount of toy matrix is ingested than when the toy is only sucked on.

Human exposure models such as CONSEXPO are currently being used in risk assessment practice to estimate internal exposure after ingestion or inhalation or dermal contact with a contaminated product. CONSEXPO is a mathematical, computer model to assess and estimate the exposure and uptake of contaminants from consumer products including toys (Bremmer and Van Veen 2002; Van Veen 2001). It was concluded that in many cases too little information was available that describes the migration of contaminants from the toy for a reliable exposure assessment. The experimental results of the in vitro digestion models that are described in the present report can be used as input for CONSEXPO for the exposure scenarios “mouthing” and “ingestion”.

In CONSEXPO a mouthing rate in $\mu g/(cm^2 \times min)$ is estimated. The duration of the mouthing phase is estimated based on the age of the child and toy type, and varies between 3 and 63 min (Bremmer and Van Veen 2002). The most straightforward approach to insert data of the in vitro digestion model into CONSEXPO is to estimate a release rate for a contaminant from toy based on experimental bioaccessibility data in saliva (suck model) for a mouthing time between 3 and 63 min. It should be kept in mind that nonlinear mobilisation of contaminant from toy matrix is possible, in which case a general release rate cannot be derived. Alternatively, a bioaccessible amount of a contaminant can be assessed for a specific mouthing time, similar to the experiments described in chapter 4.

For ingestion of a toy, it is assumed in CONSEXPO that the contaminant is completely absorbed. The data obtained with the in vitro digestion model (suck-swallow and swallow model) show that the bioaccessible fraction can be considerable smaller than 1 (between 0.001 and 0.51 in the present study). As the bioaccessible fraction always is greater than the fraction absorbed, the experimentally determined bioaccessible fraction can directly fit into the CONSEXPO model instead of the present default absorption factor 1.

Our data showed that the migration of a compound is very much dependent on the toy matrix. This implies that no general migration parameter can be estimated for one compound but that many compound / toy matrix combinations have to be tested in the in vitro digestion model to obtain a reliable dataset for CONSEXPO. Otherwise bioaccessibility has to be determined for each specific case.

6. Conclusions

Application of in vitro digestion model as a tool in risk assessment to estimate the oral exposure to contaminants from toys were studied by means of in vitro mobilisation of lead (Pb) from 4 toy matrices in the gastrointestinal tract (bioaccessibility). Three possible scenarios for the oral exposure of children to toys, i.e. sucking on the toy, sucking in combination with swallowing the toy, or immediately swallowing the toy without sucking were simulated with a suck, suck-swallow, and swallow variant of an in vitro digestion model.
The bioaccessibility of Pb from the 4 toys matrices as determined in the small intestinal compartment, where absorption takes place, was lowest for the suck model between <0.2% and 5.4%, intermediate for the suck-swallow model between 0.1% and 23%, and highest for the swallow model between 1.6% and 51%. Bioaccessibility in the mouth compartment of the suck model varied between <0.2% and 8.4%.

The main conclusions of the present study are:

- Bioaccessibility was for the present experiments considerable lower than 100%, suggesting that only a fraction of the Pb in the toy can contribute to internal exposure in man.
- Circumstances under which the toys are being handled, sucking versus swallowing, affect bioaccessibility c.q. oral bioavailability of Pb from toy matrices.
- The results illustrate the importance of the matrix on the bioaccessibility c.q. oral bioavailability of compounds as the bioaccessibility of Pb from the 4 toy matrices varied up to a factor 230 (between 0.1% and 23%) within one model.
- The reproducibility of the suck-swallow digestion model was investigated by examining the bioaccessibility data. The reproducibility of the suck-swallow model is acceptable and in some cases large, but is likely to improve when the adjustment of pH in the stomach is standardised.
- The data obtained with the in vitro digestion model can be implemented in the human exposure model CONSEXPO.
- The data demonstrate that risk assessment based on migration of Pb from toys under acid conditions is likely to overestimate oral bioavailability as bioaccessibility of Pb in the small intestinal compartment was 2- to 50-fold lower than bioaccessibility in the stomach.
- Considering the conclusions mentioned above, the in vitro digestion models appear to be a useful tool to investigate the bioaccessibility of contaminants from toys as an indicator for oral bioavailability of the contaminant.

Results obtained with the in vitro digestion models should be interpreted with care as not many contaminants and matrices have been tested so far. In addition, variables such as the amount of matrix ingested, shape and thickness of the matrix and presence of food constituents have not been examined yet. Before the digestion models can be implemented as a tool in risk assessment, the models have to be validated against the in vivo situation.

References

Altman PL, Dittmer DS (1968) Metabolism. Federation of American Societies for Experimental Biology, Bethesda, Maryland, USA

Bremmer HJ, Van Veen MP (2002) Children's toys fact sheet. Report no 612810 012. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Charman WN, Porter CJH, Mithani S, Dressman JB (1997) Physicochemical and physiological mechanisms for the effects of food on drug absorption: the role of lipids and pH. J. Pharm. Sci. 86:269-282

Dieter MP, Matthews HB, Jeffcoat RA, Moseman RF (1993) Comparison of lead bioavailability in F344 rats fed lead acetate, lead oxide, lead sulfide, or lead ore concentrate from Skagway, Alaska. J. Toxicol. Environ. Health 39:79-93

Foulkes EC, Bergman D (1993) Inorganic mercury absorption in mature and immature rat jejunum: transcellular and intercellular pathways in vivo and in everted sacs. Toxicol. Appl. Pharmacol. 120:89-95

Fox K, Zauke G-P, Butte W (1994) Kinetics of Bioconcentration and Clearance of 28 Polychlorinated Biphenyl Congeners in Zebrafish (Brachydanio rerio). Ecotoxicol. Environ. Safety 28:99-109

Freeman GB, Dill JA, Johnson JD, Kurtz PJ, Parham F, Matthews HB (1996) Comparative absorption of lead from contaminated soil and lead salts by weanling Fisher 344 rats. Fundam. Appl. Toxicol. 33:109-119

Freeman GB, Johnson JD, Killinger JM, Liao SC, Feder PI, Davis AO, Ruby MV, Chaney RL, Lovre SC, Bergstrom PD (1992) Relative bioavailability of lead from mining waste soil in rats. Fundam. Appl. Toxicol. 19:388-398

Gan L-SL, Thakker DR (1997) Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium. Advan. Drug Delivery Rev. 23:77-98

Groot ME, Lekkerkerk MC, Steenbekkers LPA (1998) Mouthing bahaviour of young children; An observational study. Household and Consumer Studies, Wageningen, Agricultural University Wageningen.

Guyton AC (1991) Textbook of medical physiology. W.B. Saunders Company, Philadelphia, USA

Hillersborg AS (2002) Final report of the work of CEN/TC52/WG9. Report no. CEN/TC 52 N851. Denmark.

Hörter D, Dressman JB (1997) Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. Advan. Drug Delivery Rev. 25:3-14

Juberg DR, Alfano K, Coughlin RJ, Thompson KM (2001) An observational study of object

mouthing behavior by young children. Pediatrics 107:135-142

Kedjarune U, Migasena P, Changbumrung S, Pongpaew P, Tungtrongchitr R (1997) Flow rate and composition of whole saliva in children from rural and urban Thailand with different caries prevalence and dietary intake. Caries Res. 31:148-154

Könemann WH (1998) Phthalate release from soft PVC baby toys; Report from the Dutch Consensus Group. Report no 613320 002. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Navazesh M, Mulligan RA, Kipnis V, Denny PA, Denny PC (1992) Comparison of whole saliva flow rates and mucin concentrations in healthy Caucasian young and aged adults. J. Dent Res. 71:1275-1278

Oomen AG (2000) Determinants of oral bioavailability of soil-borne contaminants. PhD Thesis. Utrecht University, Utrecht, The Netherlands.

Oomen AG, Rompelberg CJM, Bruil MA, Dobbe CJG, Pereboom DPKH, Sips AJAM (2003) Development of an in vitro digestion model for estimation of bioaccessibility of soil contaminants. Arch. Environ. Contam. Toxicol. 44:281-287

Oomen AG, Hack A, Minekus M, Zeijdner E, Cornelis C, Schoeters G, Verstraete W, Wiele TVd, Wragg J, Rompelberg CJM, Sips AJAM, Wijnen JV (2002) Comparison of five *in vitro* digestion models to study the bioaccessibility of soil contaminants. Environ. Sci. Technol. 36:3326-3334

Rotard W, Christmann W, Knoth W, Mailahn W (1995) Bestimmung der resorptionsverfügbaren PCDD/PCDF aus Kieselrot. UWSF-Z. Umweltchem. Ökotox. 7:3-9

Salvolini E, Mazzanti L, Di Giorgio R, Fratto G, Curatola G (1999) Changes in the composition of human unstimulated whole saliva with age. Aging Clin. Exp. Res. 11: 119-122

Strikwerda K (2002) Final report on the second saliva test with three simulants by The Convenor. Report no N 164 REV 1. The Netherlands.

Tortora GJ, Grabowski SR (1996) Principles of anatomy and physiology. Eight edition. HarperCollings Publishers Inc., Menlo Park, California, USA.

Van Veen MP (2001) CONSEXPO 3.0; Consumer exposure and uptake models. Report no 612810 011. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Versantvoort CHM, Van de Kamp E, Rompelberg CJM (In preparation) Development of an in vitro digestion model to determine the bioaccessibility of contaminants from food. Report no 320102002. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

Wienk KJH, Marx JJM, Beynen AC (1999) The concept of iron bioavailability and its assessment. Eur. J. Nutr. 38:51-75

Zeilmaker MJ, Oomen AG, De Zwart LL, Duits M, Van Eijkeren JCH, Sips AJAM (In preparation) Mathematical modeling of in vitro human bioaccessibility of lead from soil in relation to the level of contamination.