RIVM report 320102002/2004

Development and applicability of an *in vitro* digestion model in assessing the bioaccessibility of contaminants from food

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This investigation has been performed by order and for the account of the Inspectorate for Health Protection, within the framework of project 320102 (formerly 630030), Development and evaluation of *in vitro* methodologies to assess the internal exposure of man to ingested contaminants.

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Abstract

This report is the fifth report of the project V/320102 (formerly V/630030) entitled: ”Development and evaluation of *in vitro* methodologies to assess the internal exposure of man to ingested contaminants”. In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. Only a certain amount of the contaminant is bioavailable. *Bioavailability* is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation and can exert its toxic effects. Studies in animals and humans indicate that the bioavailability of compounds from food can be significantly different depending on the food source (food product), food processing or food preparation. As a consequence, a contaminant in product A can lead to toxicity whereas the *same amount* of contaminant in product B will not exert toxic effects. On the basis of this knowledge we aimed to focus our research on development of experimental tools for estimating effects of the product on bioavailability of ingested contaminants in humans.

Release of the contaminant from the ingested product in the gastrointestinal tract is a prerequisite for uptake (and bioavailability) of a contaminant in the body. The digestion processes in the human gastrointestinal tract can be simulated in a simplified manner with *in vitro* digestion models. This report describes the development of an *in vitro* digestion model allowing for measurement of release of contaminants from the ingested product as an indicator of oral bioavailability. Because food is considered a major source for exposure to many contaminants, the *in vitro* digestion model was used to study the release of contaminants from food products. This *in vitro* digestion model can also be used to examine the effects of food on the oral bioavailability of ingested contaminants from other orally ingested matrices such as soil and toys (see also RIVM-reports 320102001 and 711701012). The model could also be applied in estimation of bioavailability of bio-active (functional food) components from different food products.

Contents

Samenvatting

Voedsel is een belangrijke bron voor blootstelling aan contaminanten. Bij risicoschatting van ingeslikte contaminanten is vaak onbekend hoeveel van de contaminant wordt opgenomen in het menselijk lichaam. Alleen het deel van de contaminant dat vrijkomt uit het voedsel (bioaccesibility) en biobeschikbaar is (terecht komt in bloed, organen en weefsels), kan toxiciteit veroorzaken. De biobeschikbaarheid van een stof is in grote mate afhankelijk van het voedselproduct, waarin het zich bevindt, of van de bereidingswijze van het voedsel. Dit bemoeilijkt een accurate risicoschatting van toxische stoffen in de mens. Daarom zijn methodieken nodig, waarmee een snelle schatting van het effect van het voedselproduct op de orale biobeschikbaarheid van een stof verkregen kan worden.

Een dergelijke methodiek is gevonden in de ontwikkeling van een *in vitro* digestiemodel. Met dit model wordt het digestieproces in het maagdarmkanaal op eenvoudige wijze gesimuleerd en kan het effect van een voedselproduct op de biobeschikbaarheid van een stof onderzocht worden. Hierbij wordt het voedselproduct waarin de stof zich bevindt toegevoegd aan het model en wordt gemeten welke fractie van een stof vrijkomt tijdens het digestieproces (bioaccessibility). Alleen deze fractie is beschikbaar voor opname in het lichaam.

In het huidige rapport wordt de ontwikkeling beschreven van een *in vitro* digestiemodel dat gebaseerd is op fysiologische omstandigheden in volwassenen na het eten van een warme maaltijd. Door te eten veranderen allerlei fysiologische parameters in het maagdarmkanaal. Dit kan tot opmerkelijk verschillen in orale biobeschikbaarheid van een contaminant leiden. Dit werd geïllustreerd aan de hand van een 8-voudige toename van de bioaccessibility van benzo[a]pyrene uit bodem wanneer gevoede in plaats van nuchtere condities in het maagdarmkanaal gesimuleerd werden in het *in vitro* digestiemodel.

De toepasbaarheid van het *in vitro* digestiemodel werd onderzocht aan de hand van drie voedselproducten waarin contaminanten waren aangetroffen: cadmium in sla en radijs, aflatoxine B1 in pinda en ochratoxine A in boekweit. De bioaccessibilities waren respectievelijk ~60% en 70% voor cadmium uit sla en radijs, ~90% voor aflatoxine B1 uit pinda's en ~60% voor ochratoxine A uit boekweit.

Als een eerste kwalitatieve validatie van het *in vitro* digestiemodel, werden effecten van adsorberende materialen op de bioaccessibility van aflatoxine B1 en ochratoxine A uit voeding vergeleken met de effecten van deze stoffen in proefdieren. Van vijf van de zes combinaties adsorbent / mycotoxine waren de resultaten verkregen met het *in vitro* digestiemodel representatief voor *in vivo* gegevens in dieren. En geen van de resultaten met aflatoxine B1 en ochratoxine A in het *in vitro* digestiemodel waren in tegenspraak met *in vivo* gegevens.

Conclusies ontwikkeling *in vitro* digestiemodel. De resultaten laten zien dat het technisch haalbaar is om de bioaccessibility van contaminanten uit voedselproducten reproduceerbaar te meten. De bioaccessibility van een contaminant was afhankelijk van de matrix waarin het zich bevond, en kon beïnvloed worden door de toegepaste experimentele condities in het digestiemodel, bijvoorbeeld simulatie van nuchtere of

gevoede condities. Niet alle contaminant werd vrijgemaakt uit de voedselproducten tijdens het digestieproces. Dit impliceert dat de blootstelling van organen en weefsels aan de contaminant lager is dan de externe blootstelling en de blootstelling aan contaminant dus waarschijnlijk overschat wordt. De resultaten van de kwalitatieve validatie van het *in vitro* digestiemodel met aflatoxine B1 en ochratoxine A in aanwezigheid van adsorberende materialen tonen aan dat het *in vitro* digestiemodel een waardevol hulpmiddel kan zijn om de *in vivo* biobeschikbaarheid van stoffen te voorspellen. Voordat het *in vitro* digestiemodel gebruikt kan worden in risicoschatting moet ze verder worden gevalideerd met *in vivo* data. Toekomstig onderzoek zal zich hierop richten.

Hoe kan het *in vitro* digestiemodel bijdragen tot een beter risicoschatting? Op dit moment wordt de humane blootstelling aan een contaminant uit voeding berekend door de inname van de contaminant per product bij elkaar op te tellen. Echter de biobeschikbaarheid van de contaminant kan per product verschillend zijn. Door de bioaccessibility van de contaminant uit de verschillende voedselproducten te bepalen als mate voor de matrix afhankelijk orale biobeschikbaarheid van de contaminant, kan het *in vitro* digestiemodel tot een betere blootstellingsschatting leiden. Ook kan het *in vitro* digestiemodel als hulpmiddel gebruikt worden bij humane risicoschatting. Tot nu toe wordt bij humane risicoschatting verondersteld dat de biobeschikbaarheid van een contaminant uit een willekeurig product gelijk is aan de biobeschikbaarheid van de contaminant uit de matrix die bij toxiciteitstudies wordt gebruikt. Bij toxiciteitstudies worden veelal drinkwater, olie of gepelleteerd diervoedsel als matrices gebruikt, terwijl mensen oraal worden blootgesteld aan contaminanten uit veel verschillende voedselproducten en eventueel ook uit andere matrices. Door een relatieve biobeschikbaarheidsfaktor te bepalen (dit is de ratio van de bioaccessibility van een contaminant uit een bepaalde matrix met de bioaccessibility van de contaminant uit de matrix van de toxiciteitstudie) kan het *in vitro* digestiemodel een hulpmiddel voor humane risicoschatting zijn.

Summary

Food is considered a major source of exposure to ingested contaminants. It is often not known how much of the ingested contaminant is actually taken up in the human body. Only the fraction of the contaminant that is released from the food (bioaccessible) and is bioavailable (concentration in blood, organ and tissues) can exert toxic effects. Studies in experimental animals and humans indicate oral bioavailability of compounds to be dependent on the food product, food processing or food preparation. This hampers an accurate risk assessment of ingested toxic compounds in humans. On the basis of this knowledge we aimed to focus our research on development of experimental tools for estimating effects of the matrix on oral bioavailability of ingested contaminants in humans.

Such a method is found in the development of an *in vitro* digestion model. With this model the digestive processes in the gastrointestinal tract are simulated in a simplified manner, allowing for measurement of the release (bioaccessibility) of ingested contaminants from food as an indicator of oral bioavailability. An *in vitro* model was preferred over *in vivo* studies since it reduces the use of experimental animals and facilitates the possibilities for investigating a large number of samples and variables in a standardised setting.

This report documents the development and optimisation of an *in vitro* digestion model representative of the human physiology of the gastrointestinal tract after eating. The *in vitro* digestion model simulating fasting conditions, which has been developed previously in our laboratory to investigate the bioaccessibility of contaminants from ingested soil [RIVM-report 711701 012], was the starting point for the development of the *in vitro* digestion model simulating fed conditions. Eating food leads to physiological changes in the gastrointestinal tract, which may have the most significant impact on oral bioavailability. This was demonstrated by 8-fold increase of the bioaccessibility of benzo[a]pyrene from soil when fed conditions instead of fasting conditions were simulated in the *in vitro* digestion model. Conversion from fasted to fed conditions had not much effect on the bioaccessibilities of lead, cadmium and arsenic from soil.

Next, the *in vitro* digestion model was used in three case studies to determine:
1) bioaccessibility of cadmium from lettuce and radish cultured on three different fields contaminated with cadmium, 2) bioaccessibility of aflatoxin B1 from 9 batches of peanuts and 3) bioaccessibility of ochratoxin A from two batches of buckwheat. Considerable amounts of cadmium, aflatoxin B1 and ochratoxin A were mobilised from the food matrices during the digestion process. The respective bioaccessibilities were ~60% and ~70% for cadmium from lettuce and from radish, ~90% for aflatoxin B1 from peanut slurries, and ~60% for ochratoxin A from buckwheat.

As a first validation of the *in vitro* digestion model, the effects of adsorbent materials on the bioaccessibility of aflatoxin B1 and ochratoxin A from food were compared with *in vivo* data in experimental animals. In 5 out of 6 combinations of adsorbents and mycotoxins, the effects of the adsorbents on the bioaccessibility of aflatoxin B1 and ochratoxin A in the *in vitro* digestion model were in agreement with the effects

observed *in vivo*. None of the effects with the *in vitro* digestion model disagreed with the effects observed *in vivo*.

In conclusion, the experiments showed that it is technically feasible to reproducibly determine the bioaccessibility of contaminants from food and other ingested matrices with an *in vitro* digestion model. Bioaccessibility of a contaminant was dependent on its matrix and could be affected by the experimental conditions applied in the *in vitro* digestion model, e.g. simulating fasted or fed conditions. Not all the contaminants were released from their matrices during digestion, indicating that internal exposure (oral bioavailability) to the contaminant was lower than the external exposure (intake of the contaminant). The results of the (pre)validation of the *in vitro* digestion model with adsorbents and bioaccessibility of aflatoxin B1 and ochratoxin A, show the *in vitro* digestion model as a possible powerful tool in predicting *in vivo* bioavailability of compounds. Before the *in vitro* digestion model can be used in risk assessment, a more quantitative validation of the *in vitro* digestion model for the *in vivo* situation in humans is recommended.

How can an *in vitro* digestion model contribute to a better risk assessment? Toxicity studies use typically liquid (drinking water, oil) or food matrices (animal feed), whereas humans are exposed to contaminants from many different food products and eventually other matrices. Currently, human exposure to a contaminant in food is calculated based on the sum of the intake of the contaminant from each food product. However, the bioavailability of the contaminant from each product can be different. By determining the bioaccessibility of the contaminant from each food product as a measure of the matrix- or food-product-dependent oral bioavailability of the contaminant, the *in vitro* digestion model can be seen as an aid to better exposure assessment of contaminants from food. The *in vitro* digestion model may also be a useful tool for improving health risk assessment by determination of a relative bioaccessibility factor, i.e. comparison of the bioaccessibility of a contaminant from the ingested matrix of interest with the bioaccessibility of the contaminant from the matrix used in the toxicity studies. This relative bioaccessibility factor is likely to reflect the relative oral bioavailability and, consequently, the toxicity of the contaminant.

1. Introduction

1.1 General introduction

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. Only a certain amount of the contaminant is bioavailable. *Bioavailability* is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation and can exert its toxic effects.

Oral bioavailability of a compound can be subdivided in three constituent processes (see figure 1 in chapter 2.1):
1. Release of the compound from its matrix in the intestinal lumen (**bioaccessibility**)
2. Transport across intestinal epithelium in the body (**intestinal transport**)
3. Degradation of the compound in the liver (and intestine) (**metabolism**)

The matrix in which a contaminant is present, for example, food product or liquid can affect the fraction of contaminant that is released from its matrix during transit through the gastrointestinal tract after ingestion. The released fraction of the contaminant, the *bioaccessibility* of the contaminant, is available for intestinal absorption. The subsequent processes affecting the bioavailability of an ingested compound are transport across the intestinal epithelium and metabolism in the liver. These two processes depend mainly on compound specific properties, such as molecular weight, lipophilicity, affinity for P450 etc [Lipinski *et al.,* 1997*;* Palm *et al.*, 1996]. The matrix itself will not affect the compound specific properties, and therefore, it is unlikely that the matrix itself will have an effect on the absorption or metabolism of the contaminant [Versantvoort and Rompelberg, 2001].

Studies in experimental animals and humans suggest that oral bioavailability of compounds from food can be significantly different depending on the food source (food product), food processing or food preparation [Wienk *et al.*, 1999*;* van het Hof *et al.*, 2000]. As a consequence, the intake of a contaminant in food matrix A can lead to toxicity whereas the intake of the same amount of contaminant in food matrix B will not exert toxic effects.

Quantification of bioavailability and bioaccessibility is difficult and often hampered by complex processes comprising digestion. Accurate *in vivo* experiments in man or experimental animals can provide the best information on the (relative) bioavailability of ingested compounds [Versantvoort *et al.*, 2000]. However, an *in vitro* technique is preferred over *in vivo* studies because it reduces the need to experimental animals and the vast amount of different products/matrices demand a simple, reproducible and standardised test procedure.

The digestion processes in the gastrointestinal tract can be simulated in a simplified manner with *in vitro* digestion models [Garret *et al.*, 1999*;* Glahn *et al.*, 1996*;* Ruby *et al.*, 2001*;* Oomen *et al.*, 2002]. With such an *in vitro* digestion model, the bioaccessibility of compounds from their matrix during transit in the gastrointestinal

tract can be investigated as an aspect of oral internal exposure to the contaminant. By examining the effects of the matrix on the bioaccessibility of ingested contaminants with an *in vitro* digestion model, the exposure assessment of man to ingested contaminants can be improved.

1.2 Aim of this report

The general aim of the project is to contribute to improvement of exposure assessment of ingested contaminants by means of developing experimental tools to estimate the internal exposure of man to ingested contaminants. Food is considered a major source for exposure to many contaminants. Recently, we have developed in our laboratory an *in vitro* digestion model to simulate the digestion of contaminated soil by children under fasted conditions [Oomen *et al.*, 2003]. Experience with this *in vitro* digestion model has been acquired during several years [Sips *et al.*, 2001*;* Oomen *et al.*, 2002*;* Oomen *et al.*, 2003]. Eating food leads to physiological changes in the gastrointestinal tract, which may have the most significant impact on oral bioavailability [Charman *et al.*, 1997]. Thus, to study bioaccessibility of a contaminant from food, fed conditions instead of fasted conditions are required. Therefore, the *in vitro* digestion model for ingestion of soil contaminants by children needs to undergo some changes to mimic the physiological processes in the gastrointestinal tract of human adults after eating food.

The aim of this report is to develop an *in vitro* digestion model, which can be used to examine the effects of the food matrix on the bioaccessibility of ingested contaminants, so that human risk assessment of contaminants in food can be improved. Thereto the following issues are addressed in this report:

1) The *in vitro* digestion model should fulfill the following criteria:
   a) The model has to represent physiology of *humans*.
   b) The *last compartment* of the model is the *small intestine* as this is the absorption site of the majority of compounds.
   c) The experimental conditions should represent a *worst-case* situation, but this should be as realistic as possible. This situation may be compound-dependent.
   d) The test procedure should be *easily applicable, robust and reproducible*.
2) Simulation of fasted or fed conditions in the *in vitro* digestion model have been compared by means of the bioaccessibility of several contaminants.
3) The optimised *in vitro* digestion model was applied in three case studies to examine the technical feasibility to determine the bioaccessibility of contaminants from food.
4) (Pre)validation of the *in vitro* digestion model with *in vivo* derived data.
5) Application of the *in vitro* digestion model in health risk assessment is discussed.

1.3 Outline of the report

In chapter 2 an introduction to internal exposure after ingestion (oral bioavailability) and bioaccessibility is given. A summary of the literature review of the effects of eating food on physiological responses in gastrointestinal tract in the three compartments mouth, stomach and small intestine is given in this chapter 3. In chapter 4 variations in experimental conditions of the *in vitro* digestion model have been examined on the bioaccessibility of various contaminants (lipophilic and metals) and

different matrices in order to lead to an optimised *in vitro* digestion model simulating fed conditions.
The optimised *in vitro* digestion model was applied in three case studies to examine its technical feasibility to determine the bioaccessibility of contaminants from food. The bioaccessibility of cadmium from lettuce and radish cultured on three different fields contaminated with cadmium, the bioaccessibility of aflatoxin B1 from 9 batches of peanuts and the bioaccessibility of ochratoxin A from two batches buckwheat were determined in chapter 5.
In chapter 6, the bioaccessibilities of aflatoxin B1 and ochratoxin A in presence of adsorbent materials determined with the *in vitro* digestion model have been compared with *in vivo* data in humans and animals as a first validation of the *in vitro* digestion model.
In chapter 7, the applicability of the *in vitro* digestion model for health risk assessment of ingested contaminants from food is discussed shortly.

1.4 Framework of research on bioaccessibility of contaminants from food

The research on bioaccessibility of contaminants from food 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 to assess the internal exposure of man to ingested contaminants. Project V/320102 consists of two lines: research on the bioaccessibility of contaminants from 1) food and 2) toys. The present report describes the development an *in vitro* digestion model for fed conditions, to assess the bioaccessibility of ingested contaminants from food. In the toy part of the project, *in vitro* digestion models (suck, suck-swallow, swallow variants) have been developed that simulate the various exposure conditions handling toys (report 320102001).

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 in soil has been developed for one specific purpose: to simulate the ingestion and digestion of contaminated soil by children under fasted conditions 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 food and toy digestion models. In addition, both projects are related in the research on the validation of the models with Pb, because the first *in vitro* - *in vivo* validation is based on the bioavailability of Pb from soil under fed and fasted conditions. The *in vitro* – *in vivo* validation has been further addressed in milestone “Haalbaarheidsstudie naar validatie van het *in vitro* digestiemodel” [Versantvoort and Rompelberg, 2001], milestone “Haalbaarheidsstudie naar combinatie *in vitro* digestiemodel met *in vitro* absorptie model” [Versantvoort and Rompelberg, 2003], and milestone “Validatie *in vitro* digestiemodel *in vivo*” (to be delivered in 2004).

2. Oral bioavailability and bioaccessibility

2.1 Oral bioavailability: definition

The term oral bioavailability knows many interpretations, mostly depending on the field of research. *Oral bioavailability* is here 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 ingested. 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).

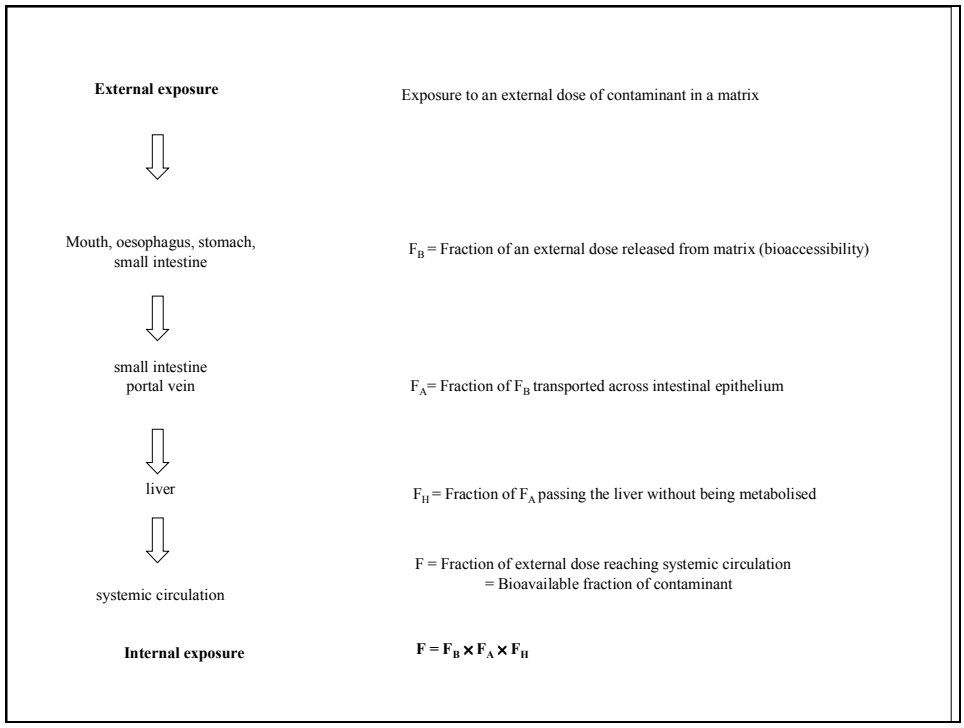

Figure 1. Schematic representation of processes determining oral bioavailability.

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 example, various dietary factors have an effect on the bioavailability of carotenoids. The bioavailability of ß-carotene from vegetables was low (~14%) compared to that of purified ß-carotene added to oil in salad dressing [van het Hof *et al.*, 2000]. The bioavailability of Fe from meat is higher than from soy or egg [Wienk *et al.*, 1999]. 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]. The effect of the matrix of ingestion on the different aspects of oral bioavailability, i.e. bioaccessibility, absorption and metabolism, is addressed in paragraph 2.2.1. and 2.2.2.

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 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 our 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 [Lipinski *et al.*, 1997*;* Palm *et al.*, 1996]. 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 [Charman *et al.*, 1997*;* 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, it can be assumed that the matrix of ingestion does not affect the transport across the intestinal epithelium or the metabolism in the liver.

2.2.3 Relative bioavailability factor

The oral bioavailability is the product of the three processes: bioaccessibility, intestinal transport and metabolism. The contribution of each individual process can not be determined in human [Versantvoort and Rompelberg, 2001]. For example, a major problem for validation of the *in vitro* digestion model against the *in vivo* situation is that different end-points are considered *in vivo* and *in vitro*. In the digestion model the fraction of the administered dose available for absorption is determined. To determine the fraction available for absorption *in vivo* samples should be taken in the small intestine at different sites and time-points, which is generally not achievable. Therefore, blood concentrations, urine excretion and or animal performance (e.g. body gain weight, feed intake, mortality) are taken as endpoints for bioavailability of (toxic) compounds. However, by comparising the internal exposure of a compound from two different matrices (e.g. drinking water vs food) or two different physiological conditions (fasted vs fed), a *relative oral bioavailability factor* can be determined.

How could the *relative bioavailability factor* be determined?
This has been described extensively in milestone "Haalbaarheidsstudie naar validatie van het *in vitro* digestiemodel" [Versantvoort and Rompelberg, 2001] and will addressed here shortly. Oral bioavailability (F) of an ingested compound is the resultant of the three steps: bioaccessibility (Fb), transport across the intestinal epithelium (Fa), and metabolism (Fh) (see figure 1).

$$F = Fb \times Fa \times Fh \quad (1)$$

The *relative bioavailability factor* is defined as

$$F_{relative} = F_{matrix\ A} / F_{matrix\ B} \quad (2)$$

and can also be written as

$$F_{relative} = (Fb \times Fa \times Fh)_{matrix\ A} / (Fb \times Fa \times Fh)_{matrix\ B} \quad (3)$$

The bioaccessibility of a contaminant, the proportion of the ingested contaminant that has been released from its matrix during digestion in the gastrointestinal tract, is affected by its compound specific characteristics, by the matrix in which the contaminant was ingested and by the digestive processes in the gastrointestinal tract. The other two processes, intestinal transport and metabolism, are mainly affected by the compound specific characteristics of the contaminant (see chapter 2.2.2). 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.

Hence, assuming that the intestinal transport and metabolism of the compound are not affected by the matrix of ingestion the relative bioavailability factor can be written as

$$F_{relative} = (Fb_{matrix\ A} \times Fa \times Fh) / (Fb_{matrix\ B} \times Fa \times Fh) \quad (4)$$

$$= Fb_{matrix\ A} / Fb_{matrix\ B} \quad (5)$$

In that case, the difference in bioavailability of a compound from two different matrices is reflected by the difference in bioaccessibility of the compound from the two matrices.

2.3 Bioaccessibility – *in vitro* digestion models

The last decade there is an increasing interest in the use of *in vitro* methodologies to study the human oral bioavailability of compounds from soil and food [Garret *et al.*, 1999*;* Glahn *et al.*, 1996*;* Minekus *et al.*, 1995*;* Ruby *et al.*, 2001*;* Oomen *et al.*, 2002]. The mobilisation of a compound from its matrix (bioaccessibility) in the gastrointestinal tract is a dynamic process with continuously changes in physiological conditions in the gastrointestinal tract. With *in vitro* digestion models the digestion process in the gastrointestinal tract is simulated 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). This is schematically illustrated 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 is mostly only determined in the chyme of the small intestine.

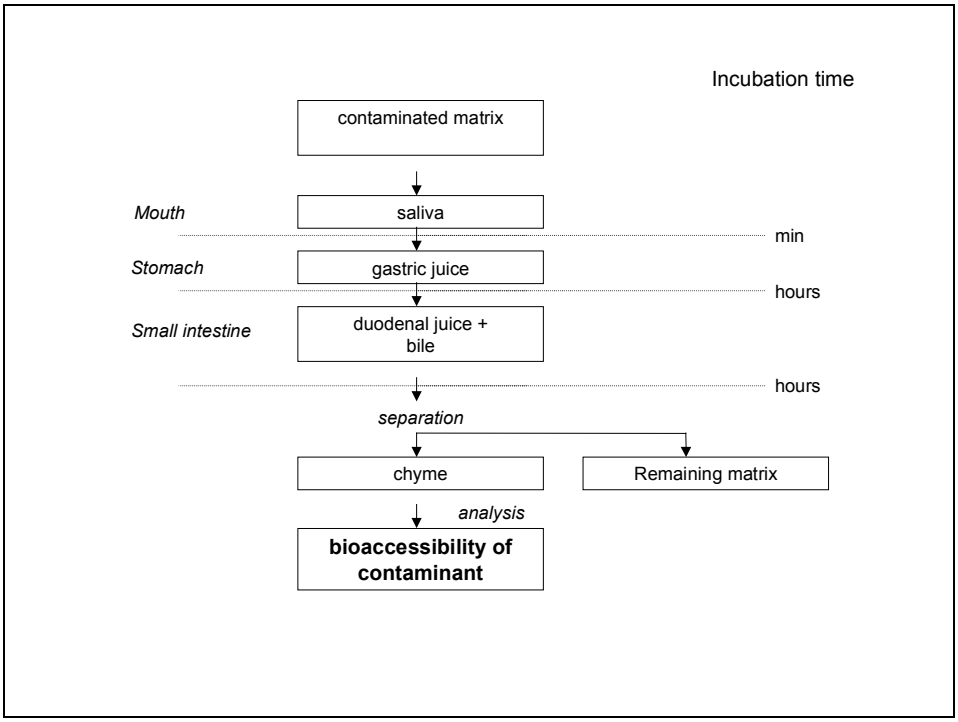

Figure 2. Schematic representation of an in vitro digestion model.
The in vitro digestion model describes a three-step procedure simulating the digestive processes in mouth, stomach and small intestine. In each compartment, the matrix is incubated at 37°C for a time relevant for the compartment. The 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. Literature research on the development of an *in vitro* digestion model simulating fed conditions.

Recently, we have developed in our Laboratory of Exposure Assessment an "*in vitro* digestion model", which consists of a three step procedure covering the digestion in mouth, stomach and small intestine [Oomen *et al.*, 2003]. This digestion model has been developed for one specific purpose: to simulate the ingestion and digestion of contaminated soil by children. This model is based on the physiology of a child under fasted conditions. Eating food leads to changes in the gastrointestinal tract due to i) secretion of gastric acid and bile and pancreatic fluids, ii) modification of gastric and intestinal motility patterns, and iii) alterations in visceral blood and lymph flow. These changes have the most significant impact on oral bioavailability. Therefore, the *in vitro* digestion model for ingestion of soil contaminants by children needs to undergo some changes to mimic the physiological processes in the gastrointestinal tract of human adults after eating food.
A summary of the literature review on changes in digestive secretions in response of food in the gastrointestinal tract and the residence time of food in the gastrointestinal tract in the three compartments mouth, stomach and small intestine is given in this chapter and described in more detail in appendix 1.

3.1 Food intake

A cooked (evening) meal was chosen as all macronutrients are ingested with a wide variety of food products. Mean intake of energy and macronutrients per cooked meal in men and women in the Netherlands are based on the food consumption measurements (VCP 1997-1998) [1998]. The mean data in Table 2 pg 52-73 of the VCP 1997-1998 were used to calculate the overall means without using a weighing factor and are shown in table 1. On average, the adult population in the Netherlands consumes equal amounts of protein and fat and a double amount of carbohydrates when eating a cooked (evening) meal with a mean caloric intake of ~800 kcal (5% percentile and 95% percentile are 299 and 1486 kcal, respectively).

Table 1. Mean intake of energy and nutrients per evening meal in men and women aged 19-65 in the Netherlands compared with two infant formulas.

	Intake cooked meal per 100g	Infant formulas Per 100g (Olvarit product number)		
	mean	451	282	282 + 2g oil
Kcal	106	67	86	103
Protein (g)	5.1	3.0	4.1	4.1
Carbohydrate (g)	9.8	7.0	11.4	11.4
Fat (g)	5.0	3.0	2.6	4.6

As a standard meal for the *in vitro* digestion model two infant formulas were chosen since the food is ready for consumption and natural products such as meat, vegetables and potatoes/rice/pasta are the source for protein, carbohydrate and fat content. The products were selected based on the caloric content and composition of the

macronutrients. The infant formulas are low in energy content: 67-86 kcal/100g compared to the mean VCP intake of 106 kcal/100 g. Product 451 has a composition of macronutrients (protein/carbohydrate/fat), which is comparable to the mean VCP consumption. However, the energy content is only 63% of the mean intake. Other food products have relatively too many carbohydrates and far too little fat compared to the adults consumption profile. By addition of 2g oil (fat), product 282 becomes comparable to the mean VCP intake by adults in respect to the energy content although the relative carbohydrate content remains high (carbohydrate: protein = 2.8 instead of 1.9).

3.2 Mouth

3.2.1 Saliva

Sour taste, chewing, and smooth objects in the mouth stimulate the saliva production. In rest condition, the flow rate is approximately 0.5 ml/min, which increases 3 to 4-fold upon stimulation with maximal flow rates of 10 ml/min [Guyton, 1991; van Amerongen *et al.*, 1994]. 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.

3.3 Stomach

3.3.1 Residence time – gastric emptying

The gastric emptying is determined by three major factors: the volume of the meal, its osmotic pressure and its the caloric content of the food. The rates of emptying of the three major foodstuffs (fat, carbohydrate and protein) are regulated so that equal numbers of calories are delivered to the duodenum in the same time (2 kcal/min) and [Hunt and Stubbs, 1975]. Fluid meals generally empty from the stomach according to first order kinetics with $T_{1/2}$ ranging from 10-60 min. Solid meals empty from the stomach according to zero order kinetics. Emptying dependent on caloric concentration ($T_{1/2}$ ranging from 60 – 277 min) but after a heavy meal completely emptying of the stomach can take up to 16 hours [Davenport, 1984].

3.3.2 Gastric pH

Fasting gastric pH is between 1.5 and 2. Upon eating a meal, the gastric pH rises temporary to pH 3-7. During gastric emptying, the gastric pH gradually declines until the fasted-state pH environment has been reestablished (usually within 1-2 h after ingestion) [Malagelada *et al.*, 1976*;* Davenport, 1984*;* Dressman *et al.*, 1990].

3.4 Small intestine

3.4.1 Bile secretion

The gallbladder contracts in a reaction to fat entering the duodenum. This results in a peak concentration of bile in the duodenum followed by a lower “constant” bile concentration. Bile, mostly produced by the liver, is secreted in the duodenum as long as there is fat in the duodenum. The gallbladder only starts refilling with bile when the stomach is almost empty [Lawson *et al.*, 1983]. The amount of bile secreted by the

gallbladder depends on the amount and the type of fat in the duodenum. In the proximal small intestine, fasting bile concentrations were in the range 1.5-5 mM (1-3 g/l chyme) and increased to 7-15 mM (5-10 g/l chyme) after eating. The bile concentration was elevated until the food was emptied from the stomach [Brunner *et al.*, 1974].

3.4.2 Pancreatic juices

Pancreatic juices are secreted in response to the presence of semi-solid chyme in the duodenum. Bicarbonate is secretion to neutralise the amount of acid entering the duodenum, whereas the enzymes start the digestion of all three major types of food. The enzyme concentration increased 2.5 to 5-fold in the duodenum in the response to food components [Brunner *et al.*, 1974].

3.4.3 Residence time

There is little difference in the mean transit times through the small intestine between the fed and fasted states. Mean small intestinal transit time is of the order of 3 hours (range 1 to 6 hours). The flow of intestinal content increases ~ 3-fold after ingestion of food [Malagelada *et al.*, 1984].

3.4.4 Intestinal pH

The pH values in the small intestine gradually increase between duodenum and ileum, from pH 5.5 to 7.5. In the duodenum, the pH after eating is lower 5-5.5 than compared to the fasted state pH 6. However, in the jejunum no major differences in pH 6-6.5 were found upon ingestion of a meal [Gray, 1996*;* Dressman *et al.*, 1998].

3.5 Volume of food and digestive fluids

The volume of fluids available in the gastrointestinal tract for a compound to dissolve in is dependent upon the volume of ingested fluids, secretions and water flux across the gut wall. About 2-3 litres are ingested per day, which is in accordance with the mean intake of adults in the Netherlands (VCP1997-1998). The ingested volume is received together with the endogenous secretions of saliva (~1L), gastric juices (~2L), pancreatic juices (~2L) and bile (~1L) by the first portion of the duodenum [Tortora and Grabowski, 1996]. These secretions total about 6 litres per day and are essential for the normal luminal digestion of foodstuffs. Most of these juices are secreted postprandially. In addition, the intestine secretes about 1-2 litres per day, to protect the epithelial cells with mucus and to improve the contact between the luminal content and the epithelial cells. This fluid is rapidly reabsorbed. In addition, much fluid (7-8 litres) is already absorbed in the small intestine. Only about 1.5 litres are presented to the colon daily, of which about 1.3 litres are absorbed, with the rest forming a component of the stool.

Thus, accounting for the absorption of digestive juices during transit, a volume ratio of 1.5 (food intake) : 1 (saliva) : 2 (gastric juice) : 2 (pancreatic juice) : 1 (bile) for the *in vitro* digestion model in project V/320102 is proposed.

3.6 Conclusions review: proposal for the *in vitro* digestion model simulating fed conditions

Based on the physiological data of the literature review, the following adaptations of the *in vitro* digestion model for ingestion of soil contaminants [Oomen *et al.*, 2003] as a result of food intake are shown in Table 2. Unless otherwise stated, it is assumed that the concentration of ions do not change from the fasted to fed state.

Table 2. Proposal for changes of the in vitro digestion model as result of food ingestion.

Digestion phase	Composition	Volume ratio	Incubation time
Food intake	Standard hot meal: protein/carbo/fat=1/2/1 (w/w/w)	1.5	
Saliva	Amylase 2x↑, Na^+↑, Cl^-↑, HCO_3^-↑, mucin↓, pH↑	1	0-5 min (fasting 5 min)
Stomach	Pepsin 2-3x↑ pH 5 (3-7) →(60 min)→ pH 2 (fasting pH 1.5-2)	2	<300 kcal: 2h 450-700 kcal: 4-7h >900 kcal: 6-20h (fasting 2h)
Bile	Standard meal: ~30 g/l Fat meal: ~60 g/l (fasting: 5 g/l)	1	2h-6h (fasting 2h)
Pancreas	Enzymes 2-3x↑, bicarbonate↑ Intestinal pH 5-7	2	

The conditions applied in the *in vitro* digestion model simulating fasted conditions are given within brackets.

4. Development and optimisation of an *in vitro* digestion model for fed conditions

In this chapter, experimental conditions in gastric and small intestinal compartment have been varied to gain insight in critical conditions in the *in vitro* digestion model that may affect the bioaccessibility of contaminants. Thereto variations in experimental conditions in gastric and small intestinal compartment have been examined on the bioaccessibility of different classes of contaminants (lipophilic and metals) and different matrices (food and soil). Based on the bioaccessibility data and the 4 defined criteria (see below), a test procedure for the *in vitro* digestion model simulating fed conditions is proposed.

1) The *last compartment* of the model is the *small intestine* as this is the absorption site of the majority of compounds.
2) The experimental conditions should represent a *worst-case situation*, but this should be as realistic as possible. This situation may be compound-dependent.
3) The test procedure should be *easily applicable, robust and reproducible*, so that application of different food products and contaminants will not affect the experimental conditions in the digestion model (too) much.

4.1 Development-starting point *in vitro* digestion model

4.1.1 Type of matrix and contaminant

Two infant formulas were used as standard food because they are representative for the mean food intake of adults at a cooked meal (see chapter 3.1) and they have already been cooked and need only to be reheated. The infant formulas were spiked with benzo[a]pyrene in sunflower oil. The reasons for choosing benzo[a]pyrene as a contaminant were mainly practical. The issue of benzo[a]pyrene in oil has been encountered several times by the Inspectorate for Health Protection and the analysis of benzo[a]pyrene in chyme was available. In addition, a soil, OECD-medium, contaminated with benzo[a]pyrene has been used previously in the *in vitro* digestion model representing fasted conditions [Sips *et al.*, 2001]. Therefore, fasted versus fed conditions in the *in vitro* digestion model could be compared as well as the effect of different matrices food versus soil on the bioaccessibility of benzo[a]pyrene.

Montana Soil containing the contaminants lead, cadmium and arsenic was also included since this matrix has been used as reference matrix in the *in vitro* digestion model for fasted conditions and this matrix consists another type of environmental contaminants i.e. heavy metals, which are also regularly encountered in food products.

4.1.2 Volume of food and digestive fluids

Accounting for the absorption of digestive juices during transit, a volume ratio of 1.5 (food intake): 1 (saliva) : 2 (gastric juice) : 2 (pancreatic juice) : 1 (bile) for the *in vitro* digestion model in project V/320102 is proposed. In practice, this means that in the intestinal compartment 9 g food, 6 ml saliva, 12 ml gastric fluid, 12 ml pancreatic fluids and 6 ml bile are mixed.

Calculated on basis of the daily volume intake of food and fluids compared to the secretion of gastrointestinal juices (chapter 3.2.4), 9 g food should be used to simulate the eating of a cooked meal. Besides simulation of a cooked meal, we aim to develop a standard fed condition in the *in vitro* digestion model to which the food product of interest (or other matrix) can be added without too much interference with the experimental conditions of the *in vitro* digestion model. Therefore, 4.5 g of the infant formulas is being used to create a standard fed condition, to which the food product of interest (or other matrix) can be added.

4.1.3 pH drift in the gastric compartment

To simulate the initially higher gastric pH 4-5 after eating (chapter 3.2.2), first 3 ml of gastric juice was added to the mixture of food and saliva. The gastric pH was 4.2 ± 0.3. This mixture was then incubated for 1 hour. Thereafter another 9 ml of gastric juice was added to lower the gastric pH to 2.0 ± 0.3 and again incubated for 1 hour. Addition of other matrices such as Montana Soil increased the pH further to pH 2.5–3.

On the other hand when a contaminated matrix is ingested an hour after eating a meal ("half empty stomach"), the pH is the stomach is already low. Since it is also more convenient to add the 12 ml gastric juice at one step, the effect of addition the gastric juice in one or two steps was determined on the bioaccessibility of benzo[a]pyrene from OECD-medium and on the bioaccessibility of lead, cadmium and arsenic from Montana Soil. Addition of the gastric juice in one or two steps had no effect on the bioaccessibility of benzo[a]pyrene from OECD-medium or on the bioaccessibility of lead, cadmium and arsenic from Montana Soil (data not shown). Therefore, in all further experiments 12 ml gastric juice was administered in one step.

4.1.4 Handling of the chyme

Today, various *in vitro* digestion models have been developed to study the bioavailability / bioaccessibility of compounds from either food or soil matrices. A comparison of the *in vitro* digestion model for fasted conditions to other digestion models was performed in a round-robin study in which the bioaccessibility values of arsenic, cadmium, and lead of three different soils were compared among five different *in vitro* digestion models [Oomen *et al.*, 2002]. Not only the composition of the digestive fluids and the incubation time of the *in vitro* digestion models varied but also the handling of the chyme. Before the bioaccessibility of the compound in the chyme is determined, the chyme and matrix are separated (see figure 2). Chyme is centrifuged, or filtrated or dialysed for separation from the matrix [Versantvoort and Rompelberg, 2003]. These different manners of separation between *in vitro* digestion models can have profound effects on the bioaccessibility of compounds [Oomen *et al.*, 2002*;* Garrett *et al.*, 1999] and may hamper the comparison of the bioaccessibility values from one *in vitro* digestion model to another. Compounds, which form complexes with proteins or with mixed bile salt micelles are likely to be less bioaccessible after dialysis as was shown for the bioaccessibility of iron from different food products [Glahn *et al.*, 1996*;* Jovani *et al.*, 2001]. However, mixed bile salt micelles not only increase the solubility of very lipophilic compounds (and lead) [Oomen, 2000] but also increase the transport of very lipophilic compounds through the intestinal epithelium [Charman *et al.*, 1997*;* Colburn *et al.*, 1985*;* Friedman and Nylund, 1980*;* Oomen, 2000]. Therefore, the bioaccessibility of lipophilic compounds after dialysis may underestimate the fraction of lipophilic compound that is available

for transport across the intestinal epithelium. By centrifugation or filtration the mixed bile salt micelles remain in the chyme and the bioaccessibility of lipophilic compounds such as benzo[a]pyrene is likely to be higher. We have chosen to separate the matrix and chyme by centrifugation at low speed because we assume that all molecules mobilised from their matrix are in principle available for transport across the intestinal epithelium and thus represents a (realistic) worst-case situation.

Thus, it should be kept in mind that comparison of absolute bioaccessibility values obtained with *in vitro* digestion models from different laboratories may be hampered by differences in experimental conditions of the *in vitro* digestion models including differences in separation of the chyme from the matrix (thus differences in determination of bioaccessibility). Nevertheless, a comparison of bioaccessibility of a compound from one matrix to another matrix is often consistent among different digestion models [Oomen *et al.*, 2002*;* Glahn *et al.*, 1996*;* Jovani *et al.*, 2001]. Hence, the *in vitro* digestion model is a promising tool for estimating the effect of the ingested matrix on bioaccessibility and thus on oral bioavailability (internal exposure) of a compound.

4.1.5 Experimental starting point for *in vitro* digestion model representative for fed conditions

The general set-up of the digestion model is as follows: the digestion starts by introducing 6 ml saliva to 4.5 gram (contaminated) standard meal. For comparison of fasted and fed conditions other matrices like 0.5 gram OECD-medium or 0.4 g Montana Soil were added. Then 12 ml of gastric juice is added, and the mixture is rotated head-over-heels for 2 hours at 55 rpm. Finally, 12 ml of duodenal juice and 6 ml bile 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 is done in a head-over-heels 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).

Compared to the composition of the digestive fluids of the fasted *in vitro* digestion model [Oomen *et al.*, 2003], the composition of saliva, gastric juice, bile and pancreatic juice is changed according to table 2. The digestive juices can be prepared the day before the actual digestion experiment. Before an experiment it is checked if the pH of the chyme (all juices together in the appropriate ratios) is pH >5.

4.2 Optimisation of *in vitro* digestion model: Research on effects of several variables on bioaccessibility

The effect of variations in experimental conditions in stomach and small intestine within the physiological windows have been studied on the bioaccessibility of benzo[a]pyrene, lead, cadmium and arsenic from two standard meals and from two soils. The following issues are addressed

1. Amount of food ingested
2. Variations of experimental conditions *in vitro* digestion model:
   - Incubation time of the gastric compartment
   - Incubation time of the intestinal compartment
   - Bile concentration in the intestinal compartment
   - pH in the intestinal compartment
3. Fasted versus fed conditions
4. Reproducibility of the test procedure

The experimental conditions of these variables are shown in table 3.
The residence time of food in the stomach is much dependent on the caloric content of the food. The stomach is emptied within 2 hours after eating small amounts of food whereas it can take up to 16 hours after eating a heavy meal. Therefore, the effect of prolonging the incubation time in the gastric compartment from 2 to 16 hours has been examined.
In the proximal small intestine, fasting bile concentrations are in the range 1.5-5 mM (1-3 g/l chyme) and increase to 7-15 mM (5-10 g/l chyme) after eating. The higher the fat content entering the duodenum, the more the gallbladder contracts and the higher (initial) concentration of bile in the proximal intestine [Brunner *et al.*, 1974*;* Lawson *et al.*, 1983]. In addition, the bile concentration is higher in the duodenum compared to the jejunum, whereas more absorption takes place in the jejunum than in duodenum (partly due to the longer length and transit time of the jejunum). Therefore, two bile concentrations were tested on the bioaccessibility of several contaminants.
In the duodenum the intestinal pH ~5.5 is lower than the intestinal pH ~6.5 in the jejunum and ileum pH~7. As absorption of compounds can take place along the small intestine, in general less is absorbed in the duodenum because of the short length and consequently short transit time. Therefore, two intestinal pH's have been tested pH 5.5-6, which has also been applied in the *in vitro* digestion model for fasted conditions [Oomen *et al.*, 2003] and a pH of 6.5-7, which is more representative for the pH in jejunum and ileum. The pancreas secretes bicarbonate to neutralise the amount of acid entering the small intestine [Brunner *et al.*, 1974]. Therefore, the pH of the chyme was increased by addition of 2 ml of the sodiumbicarbonate solution.
Based on the bioaccessibility data and the 4 defined criteria (see 1st paragraph chapter 4), a test procedure for the *in vitro* digestion model simulating fed conditions is proposed.

Table 3. Scheme of variables tested in the in vitro digestion model.

Type of matrix	Type of contaminant	Concentration contaminant (µg/g matrix)*	Contamination	Amount of food digested	Incubation time stomach	Incubation time intestine	pH intestine	Bile concentration (g/l)
Standard meals	Benzo[a]pyrene		Artificial	1g, 4.5g, 9g	2h and 16h	2h and 4h	5.5-6 and 6.5-7	30 and 60
IF 451		2.0						
IF 282		1.7						
OECD-medium	Benzo[a]pyrene	170	Artificial	0.6 g OECD + 4.5 g IF	2h and 16h	2h and 4h	5.5-6 and 6.5-7	30 and 60
Montana Soil	Pb, Cd, As	1162, 105, 42	Historical	0.4 g Montana soil + 4.5 g IF	2h and 16h	2h and 4h	5.5-6 and 6.5-7	30 and 60
Lettuce	Cd	0.31, 0.6, 0.73	Historical	9 g vegetable ± 4.5 g IF	2h	2h	6.5-7	30
Radish		0.055, 0.11						
Peanut slurry	Aflatoxin B1	1.5, 2.4, 3.6, 6.2, 9.8, 12.1, 13.3, 20.9, 36.0[#]	Historical	0.5 and 4.5g peanut slurry ± 4.5 g IF	2h	2h and 4h	6.5-7 and 5.5-6	30 and 60
Buckwheat	Ochratoxin A	7, 20[#]	Historical	1 and 4.5g buckwheat ± 4.5 g IF	2h	2h and 4h	6.5-7 and 5.5-6	30 and 60

* Cadmium concentration per g dry weight vegetable were 7.7, 15.0 and 16.5 µg Cd/g dry weight for lettuce and 1.1 and 2.3 µg Cd/g dry weight radish.
[#]Aflatoxin B1 and ochratoxin A concentrations were in µg/kg

4.2.1 Effect of the amount of food on bioaccessibility of B[a]P

The effect of the amount of food (and the amount of contaminant) on the bioaccessibility of B[a]P from the standard meals was studied by using 1, 4.5 and 9 gram contaminated standard meal in the digestion experiments.

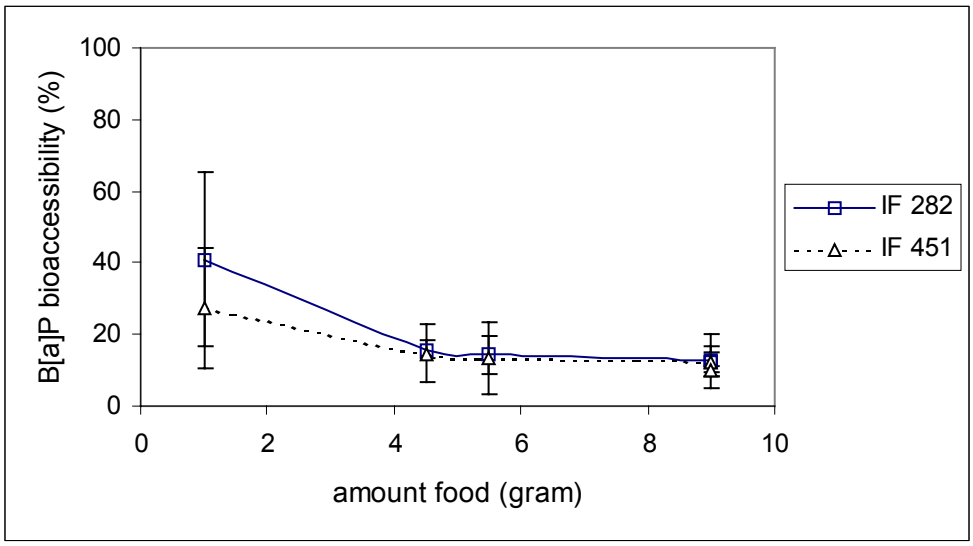

Figure 3. Bioaccessibility of B[a]P as a function of amount of food.
Data are mean ± sd of two digestion experiments with 4 replicates.

Figure 3 shows that for both standard meals the bioaccessibility of B[a]P was higher when 1 g food was used than when more food was used. At 1 gram food not only the amount of food is lower but also the amount of B[a]P is proportionally lower. By addition of 1 gram contaminated food to the standard conditions i.e. 4.5 gram not-contaminated food, we could discriminate between an effect by the amount of food or an effect by the amount of contaminant. As the bioaccessibility of B[a]P decreased when 4.5 gram not-contaminated food was included (in total 5.5 g food), the higher bioaccessibility of B[a]P was due to the low amount of food used. Apparently, the distribution of B[a]P between chyme and matrix was somewhat shifted towards the chyme when small amounts of food were used.

4.2.2 Variations of experimental conditions of the vitro digestion model

The results of variations of experimental conditions *in vitro* digestion model regarding
1. Incubation time of the gastric compartment
2. Incubation time of the intestinal compartment
3. Bile concentration in the intestinal compartment
4. pH in the intestinal compartment

are described in the next paragraphs.

4.2.2.1 Incubation time in the gastric compartment

For none of the 4 matrices, prolongation, from 2 to 16 hours, of the incubation time in the gastric compartment had an effect on the bioaccessibility of any of the contaminants (table 4). The experiments with the pH shift in the gastric compartment (chapter 4.1.3) showed that even an incubation period of 1 hour at low pH in the

gastric compartment had no effect on the bioaccessibility of benzo[a]pyrene, lead, cadmium and arsenic from their matrix in the chyme.

Table 4. Bioaccessibility (%) of contaminants in chyme: variation of experimental conditions of the in vitro digestion model.

Matrix	Contami-nant	Control	16 h stomach	4 h intestine	60 g/l bile	pH 7 intestine
IF 282	B[a]P	17 ± 2	18 ± 2	42 ± 27	36 ± 5	79 ± 15
IF 451	B[a]P	15 ± 4	16 ± 6	18 ± 5	36 ± 3	59 ± 10
OECD 282	B[a]P	25 ± 8	24 ± 4	42 ± 5	36 ± 5	43 ± 6
OECD 451	B[a]P	17 ± 4	17 ± 2	27 ± 9	33 ± 6	45 ± 7
MS 282	Pb	20 ± 5	26 ± 3	36 ± 6	25 ± 2	37 ± 3
MS 451	Pb	19 ± 3	22 ± 2	26 ± 2	18 ± 3	27 ± 4
MS 282	Cd	38 ± 5	42 ± 5	53 ± 9	44 ± 3	50 ± 5
MS 451	Cd	37 ± 5	39 ± 5	45 ± 4	39 ± 5	44 ± 6
MS 282	As	36 ± 4	39 ± 5	36 ± 6	38 ± 4	44 ± 5
MS 451	As	37 ± 5	40 ± 6	38 ± 4	38 ± 3	44 ± 7

Control conditions are: 4.5 g (contaminated) standard meal, 2 h incubation in gastric compartment, 2 h incubation in intestinal compartment with 30 g/l bile and pH 5-6. Data are mean ± sd of 2 to 9 experiments each performed in 3 to 6 replicates.

4.2.2.2 Incubation time in the intestinal compartment

Prolongation of the incubation time from 2 to 4 hours (3h is mean transit time [Malagelada *et al.*, 1984]) in the small intestine increased the bioaccessibilities of benzo[a]pyrene, lead and cadmium but not for arsenic (table 4). The increases in bioaccessibility were small (<1.6-fold increase) when standard meal 451 was used and increments in bioaccessibility of 1.4 to 2.5-fold were measured when standard meal 282 was used.

4.2.2.3 Bile concentration

Doubling the bile concentration increased the bioaccessibility of only benzo[a]pyrene while it had no effect on the bioaccessibility of the other contaminants (table 4). Benzo[a]pyrene is a very lipophilic compound and solubilisation in aqueous solutions is greatly increased by the presence of mixed bile salt micelles [Vetter *et al.*, 1985*;* Wiedemann and Kamel, 2002]. Doubling the bile concentration increases the number of micelles and thereby the fat solubilising capacity of the chyme for benzo[a]pyrene.

4.2.2.4 pH in the intestinal compartment

The increase in pH of the chyme to pH 6.5-7 had a major effect on the bioaccessibility of benzo[a]pyrene. The bioaccessibility of benzo[a]pyrene from food increased 4-fold and from OECD-medium 2-fold. The increase in bioaccessibility of lead, cadmium and arsenic from Montana Soil was less, 1.6- to 1.2-fold increase, respectively.

The structural characteristics of the mixed bile salt micelles are dependent on ionic strength and pH of the solution and on the presence of lipid digestion products [Vetter *et al.*, 1985, Charman *et al.*, 1997]. The digestion of lipids may also be improved by the increase in intestinal pH [Friedman *et al.*, 1980]. The products of lipid digestion decrease the critical micelle concentration, increase the size of the micelles and increase the solubilisation capacity of the micelles [Charman *et al.*, 1997*;* Wiedemann and Kamel, 2002]. Thus, rather than increasing the number of the mixed bile salt

micelles by doubling the bile salt concentration (see above), the solubilisation characteristics of the mixed bile salt micelles are changed by the increase in pH with a sodiumbicarbonate solution.

As the lipophilic compound benzo[a]pyrene is dependent for it solubilisation on the bile salt mixed micelles, it can be expected that the effects of increasing the pH of the chyme with sodium bicarbonate, are most pronounced on the bioaccessibility of benzo[a]pyrene. Previous studies have shown that not only lipophilic compounds interact with bile mixed micelles, but lead has been shown to form complexes with proteins and bile in the chyme [Oomen, 2000]. Apparently, the partition of lead and cadmium between matrix and chyme was thus affected that the bioaccessibility of lead and cadmium increased to a small extent.

4.2.3 Fasted versus fed conditions

The conversion from fasted to fed state was compared for the bioaccessibility of soil contaminants. The bile concentration simulating fed conditions is 5-fold increased and lipid digestion products are present, thus, the fat solubilisation capacity of the chyme is increased compared to the fasted conditions in the *in vitro* digestion model. Therefore, it was anticipated that the bioaccessibility of the very lipophilic benzo[a]pyrene would be highest in chyme with the best fat solubilisation capacity (fed contiditions). Figure 4 shows that changing the experimental conditions from simulating the fasted state [Sips *et al.*, 2001] to the fed state increased the bioaccessibility of benzo[a]pyrene from OECD-medium from 5% to 43% at intestinal pH 6.5-7, respectively.

Experimental fasted or fed conditions in the *in vitro* digestion model did not affect the bioaccessibility of lead, cadmium and arsenic from Montana Soil to a great extent. This came rather as a surprise as the *in vivo* bioavailability of lead under fasted conditions is 3- to 8-fold higher than under fed conditions [Maddeloni *et al.*, 1998*;* Graziano *et al.*, 2001*;* Dieter *et al.*, 1993*;* Freeman *et al.*, 1996]. The bioaccessibility of lead (and other heavy metals) is considerably affected by pH values in the gastrointestinal tract [Ruby *et al.*, 1996*;* Oomen *et al.*, 2002]. A low pH in the gastric compartment is required for the release of lead from its matrix [see also RIVM report 320102001]. The gastric pH is lower for the fasted conditions pH 1.5-2 compared to the fed conditions pH 2.5-3. Therefore, it was anticipated that the bioaccessibility of lead from Montana Soil was higher under fasted than fed conditions. On the other hand, *in vitro* studies have shown that lead forms complexes with proteins and mixed bile salt micelles [Oomen, 2000]. Since the concentration of these complexing agents in the chyme is higher under fed compared to fasted conditions, a higher bioaccessibility of lead may be anticipated. Apparently, the increase in complexing agents in the chyme simulating fed conditions compensate for the somewhat higher gastric pH 2.5-3. Thus, fasted or fed conditions in the *in vitro* digestion model may have profound effects on the bioaccessibility of contaminants. This effect appears to be compound specific. It can be anticipated that for lipophilic compounds fed conditions will represent worst case.

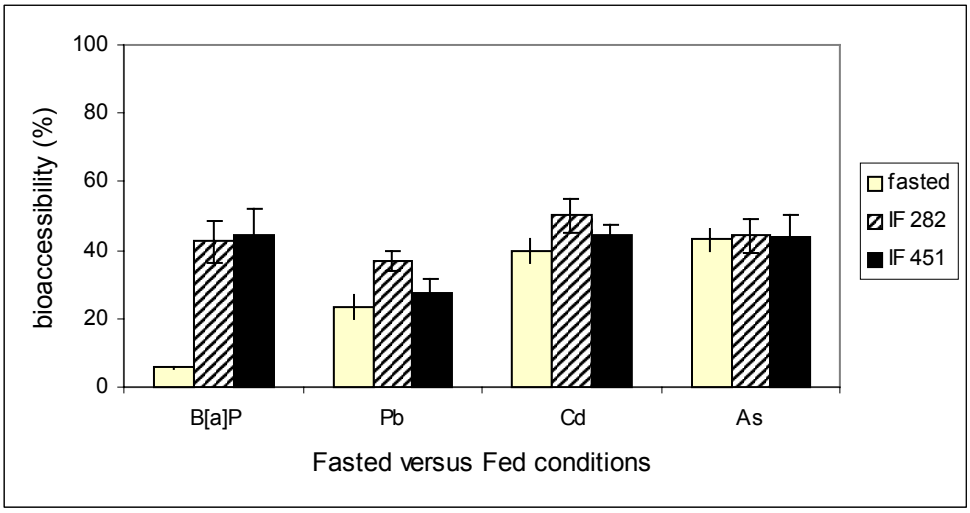

Figure 4. Comparison of fasted and fed conditions (intestinal pH 6.5-7) in the in vitro digestion model on the bioaccessibility of benzo[a]pyrene, lead, cadmium and arsenic from soil.

4.2.4 Reproducibility of the test procedure

Reproducibility of the test procedure was examined by measurements of the pH in the small intestinal compartment but also by parameters like within-day and between-day variation. To that end, standard meals and OECD-medium spiked with benzo[a]pyrene and Montana Soil were digested in 3 to 6 fold. Between-day variation was determined over a period of at least 4 days. Within-day and between-day variation was calculated by means of ANOVA. The results are shown in table 5.

The between-day variation varied from 9 % to 54% (mean 25%) and the within day variation varied from 3% to 74% (mean 17%) and the between-day variation was in general higher than the within-day variation. The within-day variation was <25%, which is acceptable for such a test procedure (digestion and analysis). Only for standard meal IF 451 the within-day variation was higher than 25% and even higher than the between-day variation. The between-day variation of bioaccessibility of benzo[a]pyrene from both standard meals as from OECD-medium was higher than for the 3 other contaminants from Montana Soil. This might be caused by a less homogeneous matrix of the standard meals and OECD-medium, which were spiked in the laboratory, while Montana Soil was historically contaminated and a certified material. In addition, the different analysis methods for benzo[a]pyrene and lead, cadmium and arsenic might contribute to a greater variation. The bioaccessibility of contaminants from Montana Soil 2711 showed the best reproducibility both at intestinal pH 5.5-6 as at pH 6.5-7. The within day variation and between day variation of Montana Soil were comparable with the *in vitro* digestion models simulating fasted conditions i.e. the suck-swallow model for toys (wdv 16% and bdv 30%, RIVM-report 320102001) and the *in vitro* digestion model for soil contaminants (wdv 18 % and bdv 26 %) [Zeilmaker *et al.,* submitted].

Table 5. Reproducibility of the bioaccessibility of various contaminants in chyme as determined by within-day and between-day variation of control conditions.

Matrix	Bioaccessibility (%) Mean ± sd	N	Within-day variation (%)	Between-day variation (%)
IF 282	17 ± 2	24	22	29
IF 451	15 ± 4	24	74	37
OECD-medium + IF282	25 ± 9	23	16	54
+ IF451	17 ± 4	23	18	46
Montana Soil + IF282, Pb	20 ± 5	11	13	37
+ IF282, Cd	37 ± 3	11	11	15
+ IF282, As	36 ± 3	11	11	13
+ IF451, Pb	20 ± 2	14	12	12
+ IF451, Cd	37 ± 3	14	15	15
+ IF451, As	37 ± 2	14	14	9
Montana Soil intestinal pH 6.5-7				
+ IF 282, Pb	39 ± 4	14	4	16
+ IF 282, Cd	51 ± 8	14	5	28
+ IF 282, As	45 ± 5	14	3	18

In future experiments different matrices and different contaminants 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. The data with Montana soil 2711 seem to be reproducible and consistent with different digestion models. 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.

4.3 Optimised *in vitro* digestion model simulating fed conditions.

The results in this chapter show that the bioaccessibility of the contaminants can be affected by the experimental conditions applied in the *in vitro* digestion model e.g. simulating fasted or fed conditions, upper part or middle part of the small intestine. From the bioaccessibility of benzo[a]pyrene, lead, cadmium and arsenic from two different standard meals and two soil matrices under different experimental conditions it was concluded that

- **Incubation time** (1 to16 hours) in the **gastric** compartment at low pH 2-3 was not a critical step for the bioaccessibility of the 4 contaminants in the small intestinal compartment.
- Experimental conditions in the **small intestinal compartment** were more critical for the bioaccessibility of the 4 contaminants:
  - **Increase of pH from pH 5.5-6 to pH 6.5-7** in the intestinal compartment greatly increased the bioaccessibility of benzo[a]pyrene and to lesser extent the bioaccessibilities of lead and cadmium
  - **Incubation time** in intestinal compartment increased the bioaccessibilities of benzo[a]pyrene and lead but less than 2-fold
  - **Doubling the bile concentration** increased the bioaccessibility of benzo[a]pyrene only.

The two different classes of contaminants (lipophilic organic compounds vs heavy metals/trace elements) were chosen in this study, as food and other matrices are frequently contaminated with these contaminants. Furthermore, it was anticipated that different factors may affect the bioaccessibility of both groups of contaminants since the oral bioavailability of very lipophilic compounds is in general higher under fed conditions whereas the oral bioavailability of lead for example is higher under fasted conditions [Graziano *et al.*, 2001*;* Charman *et al.*, 1997].

The experimental conditions at intestinal pH 6.5-7 were chosen as test procedure to simulate fed conditions in the gastrointestinal tract because

- These experimental conditions are representative for jejunum, middle part of the small intestine, where absorption of most compounds takes place.
- The bioaccessibility of all 4 contaminants was highest at pH 6.5-7 in the intestinal compartment and therefore, these conditions represent the worst-case situation for all 4 contaminants tested (criteria 3).
- At pH 6.5-7 in the intestinal compartment the bioaccessibility of benzo[a]pyrene from the standard meals was so high that is unlikely, at this pH, that the amount of food ingested, incubation time and bile concentration will increase the bioaccessibility much more. Therefore, these conditions are likely to represent the most robust conditions (criteria 4).

The test procedure is shown in figure 5.

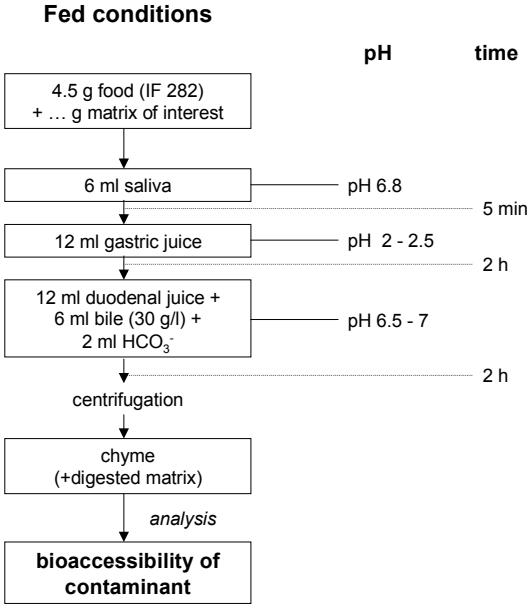

Figure 5. Schematic representation of experimental conditions of in vitro digestion model simulating fed conditions to use as test procedure.

5. Case studies: determination of bioaccessibility from contaminated food products

The technical feasibility of the *in vitro* digestion model for fed conditions was tested in three case studies by means of determination of the bioaccessibility of 1) cadmium from radish and lettuce grown on contaminated soil, 2) aflatoxin B1 from peanuts and 3) ochratoxin A from buckwheat. The experimental conditions are shown in table 3.

5.1 Cadmium in lettuce and radish.

Parts of "de Kempen" in the Netherlands and Belgium are polluted with cadmium and zinc due to past industries. In order to test whether the soil still can be used as private kitchen-gardens, radish and lettuce were cultivated on polluted soil (site 1) and at two sites which had been treated with clay (site 2) or cement (site 3).

To test the feasibility of the *in vitro* digestion test procedure, the bioaccessibility of cadmium from lettuce and radish grown on contaminated soil was determined in this case study. If it is feasible to determine the bioaccessibility of cadmium, the following issues are addressed:

1. Has the matrix cq. lettuce and radish, effect on the bioaccessibility of cadmium?
2. Has the treatment of the soil effect on the bioaccessibility of cadmium from the crops?
3. Has inclusion of a standard meal effect on the bioaccessibility of cadmium from the crops?

5.1.1 Experimental

From each site, radish (radish did not grow on untreated soil) and lettuce were collected and handled as follows: roots and tops were removed from the radish and only the nice leaves of the lettuce were used. Then, the leaves of lettuce and the radish were carefully washed and homogenates were prepared by using a blender. The cadmium concentration in the homogenate was determined by destruction and is shown in table 6. As there was not enough lettuce – cement homogenate left, the cadmium concentration in the lettuce was determined in other lettuce leaves than used for the digestion experiment. As result, the mass-balance of lettuce grown on cement treated soil was lower, 64-73%, than the mass-balance for lettuce cultivated on untreated and clay-treated soil, 87-109%.

As the concentration of cadmium in the homogenates of lettuce and radish was rather low, certainly compared to the cadmium concentration found in Montana Soil (see tables 3 and 5), we decided to use 9 gram homogenate instead of 4.5 gram. The bioaccessibility of cadmium was determined in triplicate for each homogenate in absence and presence of 4.5 gram standard meal, to examine the effect other food components on the bioaccessibility of cadmium.

As control for succession of the *in vitro* digestion procedure, Montana Soil was used as reference material. The bioaccessibilities of lead, cadmium and arsenic were consistent with the results in Table 3.

5.1.2 Results: Bioaccessibility of cadmium from lettuce and radish

The concentration of cadmium in lettuce was higher than the maximum concentration allowed for human consumption of 0.1-0.2 mg/kg (EU regulations). Similar concentrations of cadmium were found for crops cultivated on contaminated soil [Cobb *et al.*, 2000]. The concentration of cadmium in chyme could be detected in all cases. In table 6, the bioaccessibility of cadmium after digestion of lettuce and radish in presence or absence of standard meals is shown.

Table 6. Bioaccessibility of cadmium from lettuce and radish.

Matrix	Contamination level (µg Cd/ g matrix)[1]	Bioaccessibility of Cd (%)	Mass balance (%)
Lettuce untreated soil	0.60 ± 0.08	63 ± 1	109
+ standard meal		57 ± 2	96
Lettuce clay treated	0.73 ± 0.05	55 ± 2	99
+ standard meal		49 ± 2	87
Lettuce cement treated	0.31 ± 0.08	45 ± 2	73
+ standard meal		38 ± 1	64
Radish clay treated	0.11 ± 0.01	66 ± 2	124
+ standard meal		63 ± 2	117
Radish cement treated	0.055 ± 0.003	69 ± 6	113
+ standard meal		74 ± 2	125

The data are mean ± sd of one experiment in triplicate.
[1] Cadmium concentration per g dry weight crops were 15.0, 16.5 and 7.7 µg Cd/g dry weight for lettuce, respectively, and 2.3 and 1.1 µg Cd/g dry weight radish.

The results from lettuce grown on soil treated with cement will not be taken into account because the bioaccessibility is likely to be underestimated because the contamination level was not determined in the same homogenate (see 5.1.1.) and the mass balance was clearly lower than the mass balance of the other samples.

A considerable amount of cadmium, but not all, was mobilised from both lettuce and radish during the digestion process. The bioaccessibility of cadmium from lettuce seemed to be slightly lower, between 49% and 63%, than the bioaccessibility from radish between 63% and 74%.

The bioaccessibility of cadmium was similar in presence or absence of 4.5 gram standard meal during the digestion procedure.

Treatment of the soil had no effect on the mobilisation of cadmium from lettuce and radish although lettuce and radish cultivated on cement-treated soil accumulated less cadmium than clay-treated or untreated soil.

5.2 Aflatoxin B1 in peanut slurries

Peanuts are regularly contaminated with aflatoxins at higher concentrations than the maximum levels for human consumption or animal feedings, 2 and 8 µg/kg (EU regulations), respectively. To test the feasibility of the *in vitro* digestion test procedure, the bioaccessibility of aflatoxin B1 from peanut slurries was determined in this case study. The peanut slurries were obtained from the Inspectorate for Health

Protection of Rotterdam, that sampled peanuts and prepared and analysed the peanut slurries for presence of aflatoxin B1 on a routine basis. The sampled lots were intended for the Dutch market. Aflatoxin B1 level of the sampled peanut lots were between 1.5 and 36 µg/kg.

The following issues were addressed:
1. Has the amount of ingested peanut slurry effect on the bioaccessibility of aflatoxin B1?
2. Has the contamination level effect on the bioaccessibility of aflatoxin B1?
3. Has inclusion of a standard meal effect on the bioaccessibility of aflatoxin B1 from peanut slurry?
4. How robust are the results with the *in vitro* digestion model? Have experimental conditions such as doubling the bile concentration, lower intestinal pH and longer incubation tome in the small intestinal compartment effect on the bioaccessibility of aflatoxin B1 from peanut slurry?
5. What is the reproducibility of measurement of the bioaccessibility of aflatoxin B1?

5.2.1 Experimental

Sampling of the peanuts and analysis of aflatoxin B1 in the peanut samples was carried out by the Inspectorate for Health Protection of Rotterdam according to European regulations. In short, from the peanuts a slurry was prepared by homogenization of 1 kg dopped peanuts with 1.5 liter water and 40 g sodium chloride for 15 min.

Determination of the bioaccessibility of aflatoxin B1 from the peanut slurries was as described in chapter 4.3. Application of 0.5 g and 4.5 g peanut slurry in the *in vitro* digestion model corresponds to the consumption of approximately 10 and 100 g peanuts, respectively.
The digestion started by introducing 6 ml saliva to 4.5 gram or 0.5 gram peanut slurry. This was incubated for 5 min. Then 12 ml of gastric juice is added, and the mixture was incubated for 2 hours at 55 rpm. Finally, 12 ml of duodenal juice, 6 ml bile and 2 ml sodium bicarbonate were added simultaneously, and the mixture was rotated for another 2 h. The pH of the chyme was determined once more. 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) in which the concentration of aflatoxin B1 was determined.
As control for succession of the *in vitro* digestion procedure, Montana Soil was used as reference material. The bioaccessibilities of lead, cadmium and arsenic were consistent with the results in Table 3.

After purification of the chyme by liquid-liquid extraction procedures, followed by an immuno-affinity column, aflatoxin B1 and ochratoxin A could be detected simultaneously by HPLC analysis. Lower limit of quantitation were ~2 pg/ml for aflatoxin B1 and ~10 pg/ml for ochratoxin A [Sizoo and van Egmond, 1997].

In some experiments a food-mix of peanut slurry (0.5 g), buckwheat (1 g) and standard meal (4.5 g) was prepared to study the effect of a standard meal on the

bioaccessibility of aflatoxin B1 and ochratoxin A at the same time. This food-mix was also used to determine the reproducibility of bioaccessibility measurements.

5.2.2 Results: bioaccessibility of aflatoxin B1 from peanut slurries

5.2.2.1 Effect of contamination level

One of the factors potentially affecting bioaccessibility is the level of contamination. The bioaccessibility of aflatoxin B1 from 9 peanut slurries ranging from 0.6 to 14 µg/kg aflatoxin B1 (contamination level peanuts 1.5-36 µg/kg) in the chyme was determined.

Aflatoxin B1 was almost completely mobilised from the peanut slurries during digestion. A more or less constant bioaccessibility percentage of 90% at each contamination level was found. The mean bioaccessibility of all 9 batches is depicted by the line in figure 6 A. The concentration of aflatoxin B1 in the chyme of the two highest contaminated peanut slurries was higher than those in the calibration curve, therefore, a smaller volume of chyme was used for analysis. Alterations in the analysis might contribute to a higher variation in the two highest contaminated slurries. Therefore, these two peanut slurries have been excluded in calibration of the dose response relationship. In figure 6B, a linear dose proportional relationship between the amount of aflatoxin B1 in the peanuts and the amount of aflatoxin B1 mobilised from the peanut slurry in the chyme is demonstrated for the peanut slurries.

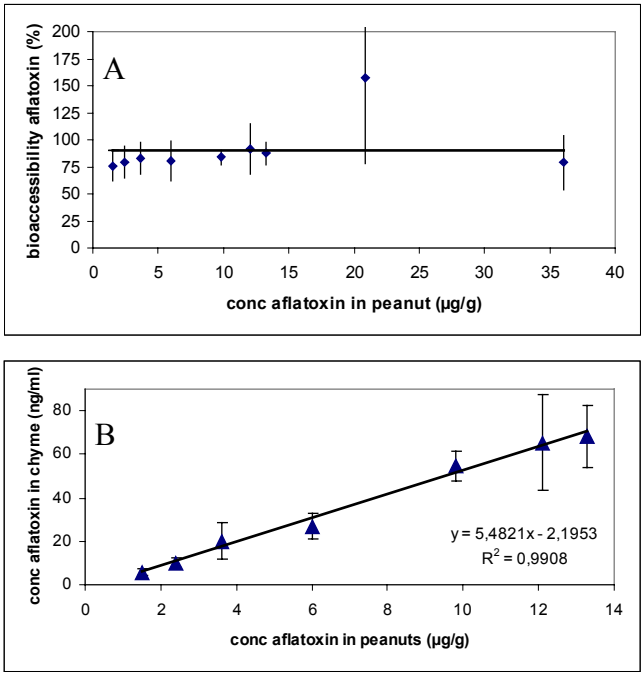

Figure 6. The amount of aflatoxin B1 released in chyme after digestion against the amount of aflatoxin B1 in peanut samples.

In figure A, bioaccessibility of 9 batches of peanut slurries are shown. Data are mean ± sd of one experiment in triplicate. Mean bioaccessibility of all 9 batches is shown by the line. In figure B, a linear dose-response relation is shown for the 7 lowest contaminated batches of peanuts.

5.2.2.2 Effect of addition of a standard meal

To examine the effect of other food components on the bioaccessibility of aflatoxin B1 from peanut slurries, a food-mix was prepared. Food-mix 1 consisted of 4.5 g standard meal, 0.5 g peanut slurry from peanuts contaminated at 6 µg/kg and 1 g buckwheat contaminated with ochratoxin A at 20 µg/kg. The bioaccessibility of aflatoxin B1 from 6 g food-mix was compared with the bioaccessibility of aflatoxin B1 from 0.5 g corresponding peanut slurry.

The results in table 7 show that the bioaccessibility of aflatoxin B1 was slightly higher when a standard meal was added, however, this difference was not significant.

Table 7. Bioaccessibility of aflatoxin B1 from peanut slurry.

Sample	Amount of aflatoxin B1 in digestion model (ng)	Bioaccessibility (%)
2 g food-mix 1	1	111 ± 13
6 g food-mix 1	3	104 ± 11
0.5 g peanut slurry (6 µg/kg)	3	80 ± 18
4.5 g peanut slurry (6 µg/kg)	27	81 ± 18

Effect of addition of standard meals and effect of amount of food ingested on the bioaccessibility of aflatoxin B1. Data are means ± sd of one or two experiments in triplicate.

5.2.2.3 Effect of amount of ingested food

Next the amount of food in the digestion model was varied: 2 and 6 gram food-mix, and 0.5 and 4.5 gram peanut slurry (6 µg/kg peanuts) was used.
As shown in table 7, the amount of food had no effect on the bioaccessibility of aflatoxin B1 from peanut slurries.

Application of 0.5 g and 4.5 g peanut slurry in the *in vitro* digestion model corresponds to the consumption of approximately 10 and 100 g peanuts, respectively.

5.2.2.4 Robustness of the digestion model

To test the robustness of the digestion model, experimental conditions such as a lower pH in the small intestinal compartment (representative for duodenum), prolonged incubation time in the small intestinal compartment (4h instead of 2h), and doubling the bile concentration were varied.
None of these variations had an effect on the bioaccessibility of aflatoxin B1 from food-mix 1 indicating that the test system is robust.

5.2.2.5 Reproducibility of determination of bioaccessibility

Reproducibility of the test procedure was examined by determination of within-day and between-day variation by means of ANOVA of the bioaccessibility of aflatoxin B1 from food-mix 1 over 3 independent digestion experiments.

Within-day variation was 12% and between-day variation was 11%. Thus, the reproducibility of bioaccessibility measurement of aflatoxin B1 was good and comparable with the variation found with other contaminants (chapter 4.2.4).

5.3 Ochratoxin A in buckwheat

Recently, investigations have been carried out on the occurrence of ochratoxin A in grain for human consumption and in various types of fodder and grain for animal consumption [Sizoo and van Egmond, 1997]. The sampled lots were intended for the Dutch market. Two batches of buckwheat were encountered to be contaminated with ochratoxin A at concentrations of 7 and 20 µg/kg, which is higher than the maximum levels for human consumption 3-5 µg/kg (EU regulations). The bioaccessibility of ochratoxin A from buckwheat was determined in this case study.

To test the feasibility of the *in vitro* digestion test procedure, the bioaccessibility of ochratoxin A from buckwheat was determined in this case study. The following issues were addressed:

1. Has the amount of buckwheat effect on the bioaccessibility of ochratoxin A?
2. Has the contamination level effect on the bioaccessibility of ochratoxin A?
3. Has inclusion of a standard meal effect on the bioaccessibility of ochratoxin A from buckwheat?
4. How robust are the results with the *in vitro* digestion model? Have experimental conditions such as doubling the bile concentration, lower intestinal pH and longer incubation tome in the small intestinal compartment effect on the bioaccessibility of ochratoxin A from buckwheat?
5. What is the reproducibility of the bioaccessibility of ochratoxin A?

5.3.1 Experimental

Determination of the bioaccessibility of ochratoxin A from buckwheat was carried out according to the test procedure as described in chapter 4.3. The digestion started by introducing 6 ml saliva to 4.5 gram or 1 gram buckwheat. This was incubated for 5 minutes. Then 12 ml of gastric juice is added, and the mixture was incubated for 2 hours. Finally, 12 ml of duodenal juice, 6 ml bile and 2 ml sodium bicarbonate were added simultaneously, and the mixture was rotated for another 2 hours. The pH of the chyme was determined once more. 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) in which the concentration of ochratoxin A was determined.
As control for succession of the *in vitro* digestion procedure, Montana Soil was used as reference material. The bioaccessibilities of lead, cadmium and arsenic were consistent with the results in Table 3.

After purification of the chyme by liquid-liquid extraction procedures, followed by an immuno-affinity column aflatoxin B1 and ochratoxin A could be detected simultaneously by HPLC analysis. Lower limit of quantitation were ~2 pg/ml for aflatoxin B1 and ~10 pg/ml for ochratoxin A.

In some experiments a food-mix of buckwheat (1 g), peanut slurry (0.5 g), and standard meal (4.5 g) was prepared to study the effect of a standard meal on the bioaccessibility of aflatoxin B1 and ochratoxin A at the same time. This food-mix was used to determine the reproducibility of bioaccessibility measurements.

5.3.2 Results: bioaccessibility of ochratoxin A from buckwheat

5.3.2.1 Effect of contamination level

One of the factors potentially affecting bioaccessibility is the level of contamination. The bioaccessibility of ochratoxin A from 2 batches of buckwheat at concentrations of 7 and 20 µg/kg in the chyme was determined.

A considerable amount of ochratoxin A, ~60%, was mobilised from the buckwheat during digestion but not all ochratoxin A. The bioaccessibility of ochratoxin A from the batch of buckwheat of 7 µg/kg showed great variation probably due inhomogeneity of the batch. It is, however, unlikely that the contamination level will have a profound effect on the bioaccessibility of ochratoxin A, as the bioaccessibility of ochratoxin A was not affected by weighing different amounts of food (and consequently different amounts of ochratoxin A) (Table 8).

Table 8. Bioaccessibility of ochratoxin A from buckwheat.

Sample	Amount of ochratoxin A in digestion model (ng)	Bioaccessibility (%)
2 g food-mix 1	7	62 ± 12
6 g food-mix 1	20	60 ± 10
1 g buckwheat (20 µg/kg)	20	53 ± 10
4.5 g buckwheat (20 µg/kg)	90	45 ± 9
4.5 g buckwheat (7 µg/kg)	32	84 ± 56

Effects of contamination level, addition of standard meal and amount of food on the bioaccessibility. Data are means ± sd of one or two experiments in triplicate.

5.3.2.2 Effect of addition of a standard meal

To examine the effect of other food components on the bioaccessibility of ochratoxin A from buckwheat, a food-mix was prepared. Food-mix 1 consisted of 4.5 g standard meal, 0.5 g peanut slurry from peanuts contaminated at 6 µg/kg and 1 g buckwheat contaminated with ochratoxin A at 20 µg/kg. The bioaccessibility of ochratoxin A from 6 g food-mix was compared with the bioaccessibility of ochratoxin A from 1 g corresponding buckwheat.

The results are shown in table 8. Addition of a standard meal had no effect on the bioaccessibility of ochratoxin A.

5.3.2.3 Effect of amount of ingested food

Next the amount of food in the digestion model was varied: 2 and 6 gram food-mix, and 0.5 and 4.5 gram buckwheat (20 µg/kg) was used. As shown in table 8, the amount of food had no effect on the bioaccessibility of ochratoxin A from buckwheat or food-mix.

5.3.2.4 Robustness of the digestion model

To test the robustness of the digestion model, experimental conditions such as a lower pH in the small intestinal compartment (representative for duodenum), prolonged incubation time in the small intestinal compartment (4h instead of 2h), and doubling the bile concentration were varied.

The bioaccessibility of ochratoxin A was not affected by any of these conditions in the digestion model showing that the test system is robust.

5.3.2.5 Reproducibility of determination of bioaccessibility

Reproducibility of the test procedure was examined by determination of within-day and between-day variation, by means of ANOVA, of the bioaccessibility of ochratoxin A from food-mix 1 over 3 independent digestion experiments.
Within-day variation was 14% and between-day variation was 30%. Thus, the reproducibility of determination of bioaccessibility of ochratoxin A was somewhat less than for aflatoxin B1 but acceptable and comparable with the variation found with other contaminants (chapter 4.2.4).

5.4 Discussion on bioaccessibility of contaminants from food products

5.4.1 Bioaccessibility of contaminants from food products

The experiments in this report showed that it is technical feasible to reproducibly determine the bioaccessibility of contaminants from food and soil matrices with the *in vitro* digestion model. The bioaccessibilities of benzo[a]pyrene, cadmium, aflatoxin B1 and ochratoxin A from food products were all moderate to high i.e. mean of 69% for benzo[a]pyrene, 62% for cadmium, 91% for aflatoxin B1 and 63% for ochratoxin A indicating that a substantial part of the contaminants is released from the food matrix but not all. It is too early to conclude that the bioaccessibility of contaminants from food is always high as not many contaminants and matrices have been tested so far. Literature data indicate that, for example, the bioavailability of ß-carotene, iron, calcium and folate was highly dependent on the type of food, and co-ingested factors as other foods or food constituents and food processing [van het Hof *et al.*, 2000*;* Wienk *et al.,*1999; Heanay, 2001*;* Gregory, 2001]. To gain insight in the usefulness of the *in vitro* digestion model for exposure assessment of contaminants from food, more contaminants should be tested and more food products per contaminant.

5.4.2 Effect of type of matrix: food versus soil

The effect of the matrix is considered by comparison of the bioaccessibilities of benzo[a]pyrene and cadmium from food versus soil (tables 4 and 6). The bioaccessibilities of benzo[a]pyrene and cadmium from food (69% and 62%, respectively) were ~1.4-fold higher than those from soil (44% and 47%, respectively), indicating that the presence of a soil matrix reduced the bioaccessibility of contaminants to some extent.

5.4.3 Concentration of contaminant

A factor potentially affecting bioaccessibility is the level of contamination. In general, a dose proportional relationship between contamination level and bioavailability is taken as basic assumption. This assumption simplifies risk assessment, since it can be assumed that regardless of the level of contamination, a constant percentage of the contaminant will be bioaccessible/bioavailable.

A dose proportional relationship was found for the contamination level and bioaccessibility of aflatoxin B1 as is shown in figure 6B. The different amounts of

benzo[a]pyrene (1.7 µg to 18 µg) and ochratoxin A (7 ng to 90 ng) in food had no effect on the bioaccessibility of benzo[a]pyrene and ochratoxin A. Furthermore, a dose proportional relationship has been found for the contamination level and bioaccessibility of lead from soil [Zeilmaker *et al.*, submitted]. However, precipitation of benzo[a]pyrene from soil was found at high concentrations [Sips *et al.*, 2001].

Thus, except for the bioaccessibility of benzo[a]pyrene from soil, a dose proportional relationship between contamination level and bioaccessibility of contaminants was found thus far. As the maximum tolerable concentrations of contaminants in food are much less than the intervention values in soil (1 ng/g food vs 40 µg/g soil for benzo[a]pyrene and 0.1-0.5 µ/g food vs 530 µg/g soil for lead), precipitation of a contaminant, as was found for benzo[a]pyrene from soil [Sips *et al.*, 2001], is not likely to occur for the release of contaminants from food because the concentration of contaminants in food is much lower. Therefore, a dose proportional relationship for the bioaccessibility and concentration of contaminants in food can be expected.

5.5 Conclusions on bioaccessibility from food products

The optimised *in vitro* digestion model was applied in three case studies to examine its technical feasibility to determine the bioaccessibility of contaminants from food. The bioaccessibility of cadmium from lettuce and radish cultured on three different fields contaminated with cadmium were 56% and 68%, respectively, the bioaccessibility of aflatoxin B1 from 9 batches of peanuts was 91% and the bioaccessibility of ochratoxin A from buckwheat was 63%.

Thus, the results showed that it is technically feasible to reproducibly determine the bioaccessibility of contaminants from food with the *in vitro* digestion model. Note that the low maximum contaminant concentration in food may demand very sensitive analytical techniques to enable the determination of the concentrations of contaminants found in chyme because the contaminant concentration is at least 10-fold diluted in the chyme compared to the concentration in food.

Furthermore, the results showed that not all of the contaminants were released from their food matrices during the digestion processes (the bioaccessibility was often less than 100%), indicating that the internal exposure to the contaminant is lower than the external exposure (intake of contaminant).

6. (Pre)validation of the *in vitro* digestion model

The *in vitro* digestion model has been based on human physiology. To test the predictive value of the *in vitro* digestion model for the *in vivo* situation, the *in vitro* digestion model should be validated with data in man. A major problem for validation of the *in vitro* digestion model against the *in vivo* situation is that different end-points are considered *in vivo* and *in vitro*. In the digestion model the fraction of the administered dose available for absorption is determined. To determine the fraction available for absorption *in vivo* the small intestine should be sampled at different sites and time-points, which is generally not achievable. Therefore, blood concentrations, urine excretion and or animal performance (e.g. body gain weight, feed intake, mortality) are taken as endpoints for bioavailability of (toxic) compounds.

Furthermore, validation of the *in vitro* digestion model with contaminants is difficult because human *in vivo* data with contaminants are scarce. *In vivo* human data on the bioavailability of lead under fasted and fed conditions are available [Maddeloni *et al.*, 1998*;* Graziano *et al.*, 2001] and might be useful for validation. However, lead shares transporters in the intestinal epithelium needed for the absorption of minerals. Therefore, the transport of lead across the intestinal epithelium is likely to be affected by the presence of food and combination of the *in vitro* digestion model with the *in vitro* intestinal transport method (Caco-2 cells) may be necessary for a good validation with the *in vivo* situation [Glahn *et al.*, 1996*;* Versantvoort and Rompelberg 2001, 2003]. More bioavailability studies with contaminants are available with experimental animals. For example, aflatoxin-induced toxicity can be inhibited by many different chemopreventive agents with multiple mechanisms of action e.g. modulation of metabolism of aflatoxin with oltipraz, phenolic antioxidants but also by reduction of exposure to aflatoxin with adsorbent materials. An “effective” adsorbent is one that tightly binds the mycotoxin in contaminated feed without dissociating in the gastrointestinal tract of the animal. The toxin-adsorbent complex passes then through the gastrointestinal tract without absorption and is eliminated via the faeces. This prevents or minimises exposure of animals to mycotoxins. Although often the efficacy of adsorption of mycotoxins has been determined by animal performance rather than by measurements of the mycotoxin concentration in blood or urine, these studies can be used to test the predictive value of the *in vitro* digestion model because less mycotoxin should be bioaccessible in the *in vitro* digestion model when it is bound to a adsorbent.

6.1 Modulators of mycotoxin bioavailability *in vivo*

A worldwide-accepted method for protecting animals against mycotoxicosis is the use of adsorbents such as HSCAS, activated charcoal and special polymers, mixed with the feed. These adsorbents have been shown to reduce toxicity of and exposure to mycotoxins in several animal species [Philips, 1999*;* Huwig *et al.*, 2001]. The following agents representative for the different classes of adsorbents i.e. Myco-AD® an aluminosilicate, activated charcoal and cholestyramine a polymer, have been selected as modulators to examine the usefulness of the *in vitro* digestion model as a predictive tool for the *in vivo* bioavailability of aflatoxin B1 and ochratoxin A. In addition, chlorophyllin has been chosen as modulator because an intervention study in human volunteers showed a 55% reduction in urinary secretion of aflatoxin

metabolites [Egner *et al.*, 2001]. It was suggested that the absorption of aflatoxin B1 was reduced due to formation of a tight complex with chlorophyllin. The mechanism of action by which the adsorbents affect the bioavailability of aflatoxin B1 and ochratoxin A, and what can be expected for the effect on the bioaccessibility of aflatoxin B1 and ochratoxin A in the *in vitro* digestion model is discussed below and summarised in Table 9.

Table 9. In vivo modulation of bioavailability of aflatoxin B1 and ochratoxin A: Predictive effect of adsorbents on the bioaccessibility of aflatoxin B1 and ochratoxin A in the in vitro digestion model.

Adsorbent	Mycotoxin	Effect	Species	Predictive effect on bioaccessibility
Activated charcoal	Aflatoxin	Yes	Poultry, goat	Decrease
	Ochratoxin	Yes	Pig, rat	Decrease
Aluminosilicate (Myco-AD®)	Aflatoxin	Yes	Rat, poultry, pig	Decrease
	Ochratoxin	No	Pig, poultry	No effect
		Yes	*In vitro* binding	
Cholestyramine	Aflatoxin	?	no information	?
	Ochratoxin	Yes	Rat, mice	Decrease
		No	Pig	
Chlorophyllin	Aflatoxin	Yes	Human, rat	Decrease or no effect
	Ochratoxin	?	no information	?

Activated charcoal.
Activated charcoal is used in man and animals as an antidote against poisoning. Activated charcoal is a non-soluble powder with an extraordinarily large surface area and pore volume that gives it a unique adsorption capacity. At high concentrations of activated charcoal (10% w/w food) activity against mycotoxicosis has been demonstrated with aflatoxin B1 in goats and ochratoxin A in pigs [Plank *et al.*, 1990*;* references in Huwig *et al.*, 2001]. At lower concentrations (<1% w/w), which show activity in aqueous solutions, no activity against mycotoxicosis was found [see references in Huwig *et al.*, 2001*;* Rotter *et al.*, 1989]. The high concentrations of activated charcoal needed *in vivo* might be due to the fact that activated charcoal is a relatively unspecific adsorbent and, hence essential nutrients are also absorbed. As the concentration of nutrients in food/feed is much higher compared to those of the mycotoxin, mostly nutrients will be adsorbed to the activated charcoal. Indeed, addition of food to the aqueous solution greatly reduced the binding capacity of activated charcoal to ochratoxin A [Rotter *et al.*, 1989].

To assess the bioaccessibility of contaminants from food products with the *in vitro* digestion model, 4.5 g standard meal is included beside the food product of interest (see figure chapter 4.3). Therefore, a high concentration of activated charcoal (10% w/w) has been chosen to use in the *in vitro* digestion model. This concentration has been proven activity against aflatoxin B1 and ochratoxin A toxicity in animals, and therefore the bioaccessibility of both aflatoxin B1 and ochratoxin A is anticipated to decrease to a great extent in presence of activated charcoal.

Aluminosilicates
Aluminosilicates are the preferred adsorbents in reduction of mycotoxicosis in animals. Aluminosilicates, mainly zeolites, hydrated sodium calcium aluminosilicates (HSCAS) and aluminosilicate-containing clays are a common anti-caking additive in animal feeds. *In vitro* binding experiments show that HSCAS can bind a broad spectrum of mycotoxins including aflatoxin B1 and ochratoxin A [Philips, 1999*;* Huwig *et al.*, 2001*;* Diaz *et al.*, 2002]. Furthermore, no nutrient interactions have been reported with HSCAS at the 0.5% level in the diet. Therefore, HSCAS seem to be more selective adsorbing agents than activated charcoal. Numerous *in vivo* studies have shown that HSCAS at 0.5-1% w/w reduce the exposure to aflatoxin B1 in various species, however, do not exert effect on other mycotoxins [Philips, 1999*;* Huwig *et al.*, 2001]. Thus *in vivo*, HSCAS are selective in adsorption of aflatoxins (and not ochratoxin A) in the gastrointestinal tract.

Myco-AD® , a formulated HSCAS clay, is a commercial mycotoxin adsorbent for all types of feed. The recommended dose is to mix it with feed at 0.25% w/w. As most *in vivo* studies have used 0.5%w/w food, this concentration was used in the *in vitro* digestion model. This concentration has been proven active against to both aflatoxin B1 and ochratoxin A in aqueous solution, *in vivo* only effective against aflatoxin B1. As the fluids used in the *in vitro* digestion model are simulating the situation *in vivo*, a decrease in bioaccessibility of aflatoxin B1 but no decrease in bioaccessibility of ochratoxin A is expected in presence of Myco- AD®.

Chlorophyllin
An intervention study in human volunteers with chlorophyllin showed a 55% reduction in urinary secretion of aflatoxin metabolites [Egner *et al.*, 2001]. Similar results have been observed in rat and rainbow trout [Kensler *et al.*, 1998*;* Breinholt *et al.*, 1995]. Although the precise mechanism of action is not yet fully understood, it is assumed that the absorption of aflatoxin B1 was reduced due to formation of a tight complex with chlorophyllin.

When this chlorophyllin-aflatoxin complex is insoluble, a decrease in bioaccessibility of aflatoxin B1 is expected in the *in vitro* digestion model. Is the complex soluble, then the bioaccessibility of aflatoxin B1 may remain unchanged. No information on combination of chlorophyllin and ochratoxin A was found.

Cholestyramine
Cholestyramine has been used for over 20 years in the clinic for reduction of low density lipoproteins and cholesterol. Cholestyramine is an anion exchange resin and binds bile acids in the gastrointestinal tract. At high cholestyramine concentrations, the absorption of fat-soluble vitamins can be decreased because the bile acids necessary for the absorption of fat-soluble vitamins, are adsorbed to the resin. Cholestyramine can act as a binding agent in *in vitro* binding assays for the mycotoxins ochratoxin A, zearalenone and fumonisins [Kerkadi *et al.*, 1999*;* Huwig *et al.*, 2001]. At 1% w/w only a very small effect of cholestryamine on the ochratoxin concentration in blood, bile and tissue was measured in pigs [reference in Huwig *et al.*, 2001]. On the other hand, cholestyramine (0.5-5% w/w) added to the diet greatly decreased the exposure to ochratoxin A and fumonisins in rats [Madhyastha *et al.*, 1992*;* Kerkadi *et al.*, 1998].

Cholestyramine was added at 5%w/w (300 mg cholestyramine per 6 g food food-mix) in the *in vitro* digestion model because this cholestyramine concentration has been shown to decrease the exposure to ochratoxin A *in vivo* [Kerkadi *et al.*, 1998]. A decrease in bioaccessibility of ochratoxin A in presence of cholestyramine is expected. The effect of cholestyramine on the bioaccessibility of aflatoxin B1 is unknown as no information on the interaction of aflatoxin B1 and cholestyramine was found.

6.2 Modulators of mycotoxin bioaccessibility *in vitro*

To assess the bioaccessibility of contaminants from food products with the *in vitro* digestion model, 4.5 g standard meal is included beside the food product of interest (see figure 5, chapter 4.3). Two food-mixes (see chapter 5.2.1) with two different batches of peanut (6 and 12 µg/g) and buckwheat (7 and 20 µg/g) were used to examine the bioaccessibility of aflatoxin B1 and ochratoxin A in presence of an adsorbent. Effects of the adsorbents on the bioaccessibility of aflatoxin B1 and ochratoxin A from food-mix 1 is shown in figure 7. The bioaccessibility found without an adsorbent added is defined as 100%. The effect of an adsorbent is expressed as

$$\text{bioaccessibility}_{\text{in presence of adsorbent}} : \text{bioaccessibility}_{\text{without adsorbent}} * 100\%.$$

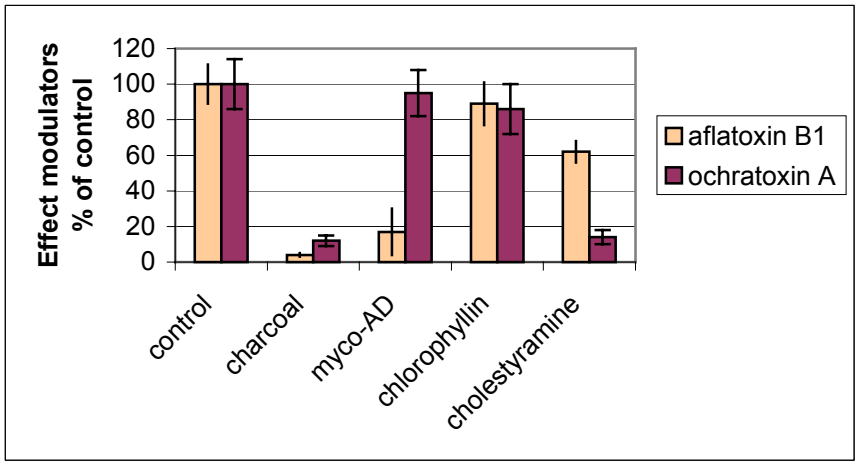

Figure 7. Effect of adsorbents on the in vitro bioaccessibility of aflatoxin B1 and ochratoxin A from food-mix 1 as determined with the in vitro digestion model.
The following amounts of adsorbents were added to 6 g food-mix: 600 mg charcoal (10% w/w food), 30 mg myco-AD® (0.5% w/w food), 3 mg chlorophyllin, and 300 mg cholestyramine (5% w/w food).

Charcoal greatly reduced the bioaccessibility of both aflatoxin B1 and ochratoxin A to less than 20% of control bioaccessibility. This was expected because at this concentration charcoal reduced the ochratoxin A levels with 50% to 80% in pig [Plank *et al.*, 1990] and mortal effects of aflatoxin in goat and mink were abolished [Huwig *et al.*, 2001*;* Bonna *et al.*, 1991].

The *aluminosilicate Myco-AD®* decreased the bioaccessibility of only aflatoxin B1 without affecting the bioaccessibility of ochratoxin A. These results are also in agreement with the effects of HSCAS on aflatoxin B1 (positive effect) and ochratoxin

A (no effect) in rat, poultry and pig [Plank *et al.*, 1990; Philips 1999; Huwig *et al.*, 2001].

Chlorophyllin is supposed to reduce the absorption of aflatoxin B1 by complexation [Egner *et al.*, 2001; Kensler *et al.*, 1998; Breinholt *et al.*, 1995]. However, addition of chlorophyllin had no significant effect on the bioaccessibility of either aflatoxin B1 or ochratoxin A. A lower absorption might be due to a lower bioaccessibility or due to a slower transport across the intestinal epithelium. Whether chlorophyllin is affecting the passage of the gastrointestinal epithelium of aflatoxin B1 could be examined by measurement of the transport of aflatoxin B1 with the *in vitro* intestinal transport method (Caco-2 cells) [Versantvoort *et al.*, 2000]. As the mechanism of action of chlorophyllin is not yet fully understood, the results of chlorophyllin on the bioaccessibility of aflatoxin B1 can not considered as positive or negative for the validation of the *in vitro* digestion model.

Cholestyramine decreased the bioaccessibility of aflatoxin B1 to a small extent and greatly reduced the bioaccessibility of ochratoxin A to less than 20% of the control bioaccessibility. This is in agreement with *in vivo* data in rats where cholestyramine reduced the bioavailability of ochratoxin A 2- to 5-fold [Madhyastha *et al.*, 1992; Kerkadi *et al.*, 1998].

Effects of the adsorbents on the bioaccessibility of aflatoxin B1 and ochratoxin A from food-mix 1 and food-mix 2 were the same (see Table 10). Furthermore, the effects of the adsorbents were also reproducible as the standard deviation of the two experiments in triplicate (n=6) is small.

Table 10. Effect of adsorbents on the in vitro bioaccessibility of aflatoxin B1 and ochratoxin A.

	Bioaccessibility % of control			
	Aflatoxin B1		Ochratoxin A	
Adsorbent	Food-mix 1	Food-mix 2	Food-mix 1	Food-mix 2
Control	100 ± 11	100 ± 8	100 ± 14	100 ± 6
Charcoal	4 ± 1	2 ± 1	12 ± 3	16 ± 5
Myco-AD®	17 ± 13	12 ± 8	95 ± 13	107 ± 11
Chlorophyllin	89 ± 12	93 ± 8	86 ± 14	94 ± 21
Cholestryamine	62 ± 6	60 ± 7	14 ± 4	13 ± 5

Data are mean ± sd of 2 digestion experiments each performed in triplicate. The following amounts of adsorbents were added to 6 g food-mix: 600 mg charcoal (10% w/w food), 30 mg myco-AD® (0.5% w/w food), 3 mg chlorophyllin (chlorophyllin : aflatoxin = >100000 : 1), and 300 mg cholestyramine (5% w/w food).

In conclusion, in 5 out of 6 combinations of adsorbents and mycotoxins the effects of the adsorbents on the bioaccessibility of aflatoxin and ochratoxin in the *in vitro* digestion model were in agreement with the effects observed *in vivo.* And none of the effects with the *in vitro* digestion model were in disagreement with the effects observed *in vivo*.

6.3 Conclusions

A first validation of the *in vitro* digestion model has been presented in this chapter. The bioaccessibilities of aflatoxin B1 and ochratoxin A in presence of 4 different adsorbents were in 5 out of 6 situations predictive for the *in vivo* situation. The sixth case showed no effect of chlorophyllin on the bioaccessibility of aflatoxin B1 whereas *in vivo* a 2-fold reduction was observed in humans. However, the mechanism by which chlorophyllin reduces aflatoxin B1 bioavailability *in vivo* are not fully understood and when the transport of aflatoxin B1 across the intestinal epithelium is decreased in presence of chlorophyllin, no effect on the bioaccessibility of aflatoxin B1 can be expected. Thus, the results with adsorbents on the bioaccessibility of aflatoxin B1 and ochratoxin A show that the *in vitro* digestion model can be a powerful tool to predict *in vivo* bioavailability of compounds.

Before the *in vitro* digestion models can be implemented as a tool in risk assessment, a more quantitative validation of the *in vitro* digestion model for the *in vivo* situation in man is recommended. As human *in vivo* bioavailability data of contaminants are rare, studies with vitamins or minerals such as ß-carotene, iron, calcium and folate from different food sources or food processing could be useful because *in vivo* studies in humans are available of these compounds.

7. Applicability of the *in vitro* digestion model in risk assessment

The general aim of the project is to contribute to improvement of exposure assessment of ingested contaminants by means of developing an experimental tool to estimate the internal exposure of man to ingested contaminants. The last decade there is an increasing interest in the use of *in vitro* methodologies to study the human oral bioavailability of compounds from soil and food [Garret *et al.*, 1999*;* Glahn *et al.*, 1996*;* Minekus *et al.*, 1995*;* Ruby *et al.*, 2001*;* Oomen *et al.*, 2002]. 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 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 is mostly only determined in the chyme of the small intestine. Sometimes the bioaccessibility is not determined directly in the chyme but after dialysis [Glahn *et al.*, 1996*;* Minekus *et al.*, 1995]. As described in chapter 4.1.4 this may influence the bioaccessibility of compounds.

The mobilisation of a compound from its matrix (bioaccessibility) in the gastrointestinal tract is a dynamic process with continuously changes in physiological conditions in the gastrointestinal tract. Most of the models are static models, which means that transition of one compartment to the other is instantaneously. The model of TNO introduced by Minekus *et al.* [1995] peristaltic movement is simulated and pH and water absorption are continuously monitored and controlled. This dynamic model is also a multi-compartmental model including the stomach and small intestinal parts jejunum and ileum (if required also colon) but not the mouth.

What methodology is best is strongly dependent on what information is required. For screening purposes, the model should be simple, cheap, able to handle many samples and result in overestimation of worst case situation (EPA-tests, Ruby, dissolution tests). The dynamic model can provide detailed information on the release of a compound from its matrix. This model requires advanced laboratory equipment and is therefore not easily introduced in an arbitrary laboratory setting. Furthermore, only small sized samples (homogenised) can be introduced in this model and one matrix can be analysed per day and, therefore, the model is less suitable for handling many samples.

Aiming at a test procedure to apply on a random matrix (food, consumer products, soil) for random compounds, the digestion model should easily be manipulated to mimic various conditions. The results in this project (reports 320102-001 and 320102-002) with the *in vitro* digestion models simulating fasted and fed conditions showed that the physiologically based *in vitro* digestion model is a promising tool for estimating the effect of the ingestion matrix (food, soil and toys) on bioaccessibility of contaminants. The method is based on head-over-heels rotation, which can easily implemented in a laboratory setting and showed reasonable interlaboratory reproducibility [Könemann *et al.*, 1998].

7.1 How can determination of the bioaccessibility of contaminants with an *in vitro* digestion model aid to improvement of health risk assessment?

7.1.1 Exposure assessment.

To date, exposure assessment to a contaminant from food is calculated based on the sum of intake (external exposure) of contaminant per food product rather than on the total internal exposure, whereas the internal exposure is correlated with toxicity of the contaminant. Contaminants are present in many different ingested (food) products and the total oral exposure to the contaminant is the sum of the bioavailability of the contaminant from each product. The bioaccessible fraction is considered to represent the maximum amount of contaminant available for absorption in the body, thus, the oral bioavailability can never be higher than the bioaccessibility. Hence, a bioaccessibility of less than 100% implies that the internal exposure to the contaminant is lower than the external exposure and that consequently the internal exposure to the contaminant is overestimated.

The results in this project showed that the bioaccessibility was dependent on the matrix and that not all of the contaminants were released from their matrix during digestion. The results should be interpreted with care as not many contaminants and matrices have been tested so far. Further determination of the bioaccessibility of contaminants from different food products is a next step to examine the effect of the food product on the bioaccessibility of contaminants. Also studies described in literature indicate that the oral bioavailability of a contaminant from food can be dependent on its matrix of ingestion [Dieter *et al.*, 1993*;* Freeman *et al.*,1996*;* van het Hof *et al.*, 2000*;* Wienk *et al.*, 1999*;* Gregory, 2001*;* Heanay, 2001]. Therefore, the *in vitro* digestion model could improve exposure assessment by taking into account the bioaccessibility of the contaminant from each product as a maximum measure for oral bioavailability in calculation of the total internal exposure to the contaminant (Σ(intake contaminant x Fb)per product).

Besides unintentionally presence of contaminants in food, bio-active components e.g. anti-oxidants, vitamins, iodine, minerals are added to food products. For these components, bioavailability is key to the effectiveness of the components. The *in vitro* digestion model could be used to determine the bioaccessibility as part of the bioavailability of the bio-active (functional food) component from different food products.

7.1.2 Health risk assessment.

Up till now in risk assessment, the oral bioavailability of a contaminant from an arbitrary ingested matrix (e.g. food, toy, soil) is assumed to equal the oral bioavailability from the matrix applied in toxicity studies. NOAELs and consequently ADIs for contaminants are based on matrices used in toxicity studies. In toxicity studies typically liquid (drinking water, oil) and food matrices (pelleted animal feed) are employed, whereas man is exposed to contaminants present in many different ingested food products. Studies in experimental animals and humans indicate that oral bioavailability of compounds is dependent on its matrix [Dieter *et al.*, 1993*;* Freeman *et al.*,1996*;* van het Hof *et al.*, 2000*;* Wienk *et al.*, 1999*;* Gregory, 2001*;* Heanay, 2001]. As a consequence, a contaminant in matrix A can lead to toxicity whereas the

same amount of contaminant in matrix B will not exert toxic effects. Introduction of a *relative bioavailability factor* for a contaminant, i.e. the bioavailability of the contaminant from the matrix of interest with respect to the bioavailability the contaminant from the matrix used in toxicity studies [Versantvoort and Rompelberg, 2001*;* van het Hof *et al.*, 2000], would therefore contribute to more accurate health risk assessment.

7.1.2.1 Determination of a relative bioavailability factor

Determination of a relative bioavailability factor has been described in milestone "Haalbaarheidsstudie naar validatie van het *in vitro* digestiemodel" [Versantvoort and Rompelberg, 2001] and the theory of oral bioavailability is described in chapter 2. Oral bioavailability of an ingested compound is the resultant of the three steps bioaccessibility, transport across the intestinal epithelium and metabolism. Assuming that the matrix of ingestion does not affect intestinal transport and metabolism of the compound (see chapter 2), the relative bioavailability factor of a compound ($F_{relative} = F_{matrix\ of\ interest} / F_{matrix\ tox\ study}$) is reflected by the difference in bioaccessibility of the compound from the two different matrices ($Fb_{matrix\ of\ interest} / Fb_{matrix\ tox\ study}$).

In figure 7 a schematic design how to handle the determination of the relative bioavailability factor is given. Often in toxicity studies, concentrations of the compound in urine or faeces are measured rather than the oral bioavailability of the compound. Therefore, usually the bioaccessibility of the contaminant both from the matrix of interest and from the matrix used in toxicity studies should be determined experimentally.

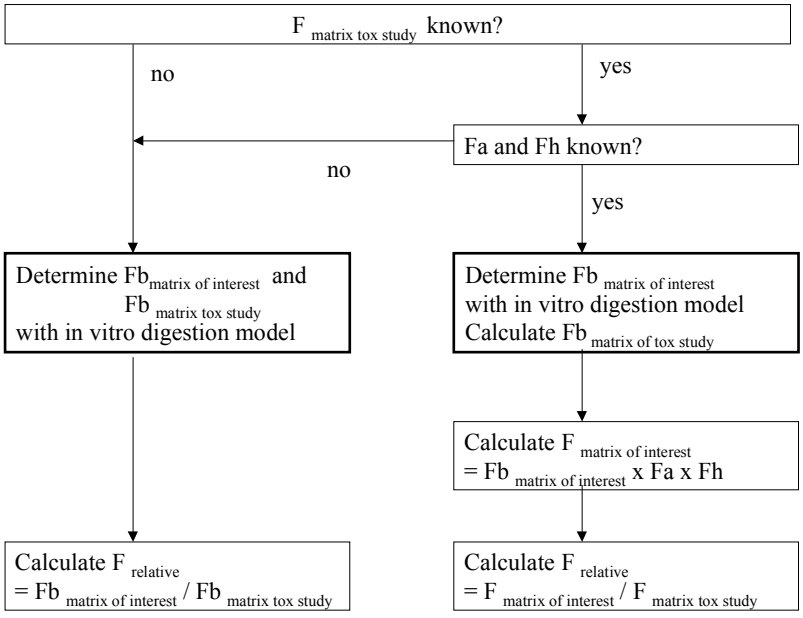

Figure 7. A schematic design for determination of the relative bioavailability factor ($F_{relative}$).

Fb, Fa, Fh are defined as the fraction of the compound that is bioaccessible, transported across the gastro-intestinal wall, and not metabolised in the liver, respectively (see figure 1).

7.1.2.2 What matrices are being used in toxicity studies?

Frequently used matrices in toxicity studies in experimental animals are drinking water (e.g. heavy metals in rats), oil (e.g. NOAEL of benzo[a]pyrene in rats has been based on gavage with soy oil), and feed (e.g. phtalate has been added to standard feed pellets in toxicity study in rats). With epidemiological studies in humans, the contaminant has often been ingested either by drinking water and/or by food (e.g. lead, cadmium in human). Notice that administration in drinking water without food supply within half an hour after administration of the drinking water, implies fasted conditions in the gastrointestinal tract whereas administration with feed and food results in fed conditions.

For a contaminant administered in solution as it is with drinking water, the bioaccessibility of the contaminant is assumed to be 100%. The contaminant does not need to be mobilised from its matrix because it is already in solution and hence available for absorption in the intestine. After ingestion of other matrices such as feed, food, soil and toys, the contaminant may be partially or totally released from their matrix during digestion in the gastrointestinal tract. Only the bioaccessible fraction is available for transport across the intestinal epithelium into the body and can contribute to internal exposure.

7.1.2.3 Matrices which have been used with the in vitro digestion model.

In this study, the bioaccessibilities of benzo[a]pyrene, cadmium, aflatoxin B1 and ochratoxin A from food products were moderate to high: 69% for benzo[a]pyrene, 62% for cadmium, ~90% for aflatoxin B1 and ~60% for ochratoxin A indicating that for most contaminants a substantial part of the contaminants is released from the food matrix but not all. It is too early to conclude that the bioaccessibility of contaminants from food is always high and that consequently comparison with the matrix used in toxicity studies is not necessary because not many contaminants and matrices have been tested so far. Furthermore, literature data indicate that, for example, the bioavailability of ß-carotene, iron, calcium and folate was highly dependent on the type of food, and co-ingested factors as other foods or food constituents and food processing [van het Hof *et al.*, 2000*;* Heanay, 2001*;* Gregory, 2001]. In this project the impact of the matrix on the bioaccessibility was illustrated by the 1.4-fold lower bioaccessibility of benzo[a]pyrene and cadmium from soil compared to food but especially by the great differences in bioaccessibility of lead (bioaccessibility varied from 1.6% to 51%) from 4 different toy matrices [RIVM-report 320102001, Oomen *et al.*, 2003]. Here it is evident that comparison of the bioaccessibility of the matrix of interest with the matrix used in toxicity studies will aid to a better exposure and hence risk assessment.

To gain insight in the usefulness of the *in vitro* digestion model for risk assessment, bioaccessibility of contaminants from matrices used in toxicity studies should be determined, for example, the bioaccessibility of benzo[a]pyrene from soy oil, lead, cadmium and arsenic in drinking water (fasted conditions) and food (fed conditions) and compared with the bioaccessibility of the contaminant from different matrices.

7.2 Conclusions

The experiments in this report and previous reports show that it is technically feasible to reproducibly determine the bioaccessibility of contaminants from food, soil and toy matrices with an *in vitro* digestion model. Only a fraction of the contaminants was released from the matrix during digestion in most cases. These results indicate that the internal exposure (oral bioavailability) to the contaminant is lower than the external exposure (intake of the contaminant) implying that exposure is overestimated.

Contaminants may be present in many different food products. The total oral exposure to the contaminant is the sum of the bioavailability of the contaminant from each product. As the bioavailability of the contaminant can be dependent on its matrix of ingestion, the *in vitro* digestion model can improve exposure assessment by taking into account the bioaccessibility of the contaminant from each product in calculation of the total exposure to the contaminant.

Furthermore, the *in vitro* digestion model could be a useful tool to improve health risk assessment by determination of a relative bioaccessibility factor, i.e. comparison of the bioaccessibility of a contaminant from the ingested matrix of interest with the bioaccessibility of the contaminant from the matrix used in the toxicity studies. This relative bioaccessibility factor is likely to reflect the relative oral bioavailability of the contaminant.

Before the digestion models can be used in risk assessment, the *in vitro* digestion models should be validated with *in vivo* derived data. The results with adsorbent materials on the bioaccessibility of aflatoxin B1 and ochratoxin A (chapter 6) show that the *in vitro* digestion model can be a powerful tool to predict *in vivo* bioavailability of compounds. Sometimes combination with an *in vitro* intestinal transport model may be necessary for a good validation. Therefore, further research should be focussed on the validation of the *in vitro* digestion models.

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Appendix 1

Physiological changes in the gastrointestinal tract in response to food. Development of an *in vitro* digestion model to mimic the fed state in adults.

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Contents

Samenvatting

Met behulp van een "*in vitro* digestiemodel", een methode waarmee op een vereenvoudigde wijze het digestieproces nagebootst wordt, kan op een relatief goedkope en snelle manier een schatting van de orale biobeschikbaarheid van een stof verkregen worden. Een *in vitro* digestiemodel kan daarom een hulpmiddel zijn bij risicobeoordeling van orale inname van contaminanten. De orale biobeschikbaarheid van contaminanten kan opmerkelijk verschillen als de contaminant op een nuchtere maag of met voeding ingenomen wordt ten gevolge van fysiologische veranderingen in het maagdarmkanaal. Contaminanten worden vaak onder wisselende condities ingeslikt, wat gevolgen kan hebben voor de toxiciteit van de contaminant. Het digestiemodel dat in ons laboratorium is ontwikkeld, is gebaseerd op de fysiologische condities van een kind onder nuchtere omstandigheden. We willen dit digestiemodel nu toepasbaar maken om de bioaccessibility van contaminanten in voeding te bestuderen. Aangezien bij de risicoschatting van contaminanten in voeding vooral naar levenslange blootstelling (chronische toxiciteit) wordt gekeken is de doelgroep niet een kind maar een volwassene. Hierdoor zal het huidige digestiemodel aangepast moeten worden om de fysiologische processen in het maagdarmkanaal van volwassenen na het eten van voeding te simuleren.

Veranderingen in de orale biobeschikbaarheid van stoffen door voeding kan het gevolg zijn van fysische en chemische interaktie tussen de stof en specifieke voedselcomponenten maar is meestal het gevolg van veranderingen in de secretie van de maagdarmsappen en/of verandering in de verblijtijftijd in het maagdarmkanaal. Aangezien de absorptie van stoffen met name plaatsvindt in de dunne darm, moet de stof al vrijgekomen zijn uit zijn matrix in de dunne darm. Daarom beperken we ons tot de fysiologische veranderingen in het traject van mond tot en met dunne darm. Dit document is bedoeld als werkdocument om op grond van literatuurgegevens tot een voorstel voor aanpassingen van het digestiemodel te komen om de fysiologische processen in het maagdarmkanaal onder invloed van voeding te simuleren.

De secretie van speeksel, maagsap, gal en pancreassap in respectievelijk de mond, maag en duodenum, wordt gestimuleerd door voedsel consumptie. Zowel de hoeveelheid geproduceerd als ook de samenstelling van de sappen verandert door voedsel consumptie. Vaak wordt juist die hoeveelheid sap geproduceerd die nodig is voor de vertering van de voeding. Andere belangrijke veranderingen zijn de verhoging van de maag pH door het eten van voeding van pH 1.5 (nuchter) tot pH 3-7 (+voeding) en de verblijftijd van de voeding in de maag. Daarentegen verandert de pH en de verblijftijd in de dunne darm nauwelijks in aanwezigheid van voeding. De veranderingen in secretie van de maagdarmsappen en de verblijftijden zijn in detail bediscusieerd in hoofdstukken 3 tot en met 6. Gebaseerd op deze literatuur gegevens is een voorstel gemaakt hoe het digestiemodel aan te passen voor voeding. Dit is in de onderstaande tabel weergegeven.

Voorstel voor aanpassingen voor het in vitro digestiemodel ten gevolge van voedsel inname.

Digestie fase	Verandering in samenstelling	Volume verhouding	Incubatie tijd
Voeding	Standaard warme maaltijd: eiwit/koolhydraat/vet=1/2/1 (w/w/w)	1.5	nvt
Speeksel	Amylase 2x↑, Na^+↑, Cl^-↑, HCO_3^-↑, mucin 2x↓, pH 7-7.4	1	0-5 minuten
Maag	Pepsine 2-3x↑ pH 5 (3-7) →(60-120 min)→ pH 2	2	2uur: <300 kcal 4-7 uur: 450-700 kcal 6-20 uur: >900 kcal
Gal	Standaard maaltijd: 8 mM Vetrijke maaltijd: 15 mM	1	2 – 4 uur
Pancreas	Enzymen 2-3x↑, bicarbonate↑	2	

1 Introduction

Implementation of comprehensive methods capable of predicting human oral absorption in risk assessment of orally ingested toxicants, would provide information that could speed up and contribute to a more rational and optimised health risk assessment. Recently, we have developed in our Laboratory of Exposure Assessment and Environmental Epidemiology an "*in vitro* digestion model", which mimics in a simplified manner the digestion processes in the gastrointestinal tract. In this way the release of a compound from its matrix in the gastrointestinal tract can be studied. Only the fraction of the compound that is released (bioaccessibility) is available for absorption in the small intestine. This *in vitro* digestion model may provide an important tool for (ad hoc) health risk assessment practice since a rapid indication of the bioaccessibility of a compound can be obtained with model. This digestion model has been developed for one specific purpose: to simulate the ingestion and digestion of contaminated soil by children (project M711701/01/FF). This model is based on the physiology of a child under fasted conditions [34]. We now like to apply the digestion model for examining the bioaccessibility of toxic compounds that have been ingested with a meal in adults. Therefore, the digestion model may need to undergo some changes to mimic the physiological processes in the gastrointestinal tract of human adults after eating a meal.

The presence of food within the gastrointestinal tract can markedly alter the oral bioavailability of compounds via changes in the rate and/or extent of absorption, presystemic metabolism, and systemic clearance of the compound. These changes can lead to variations in toxicity profiles because contaminants are often ingested under conditions of varying food and fluid intake. Physical and chemical interactions may occur between compounds and specific food components but altered postprandial absorption is generally a function of the changes associated with conversion from the fasted to the fed state. Changes due to i) secretion of gastric acid and bile and pancreatic fluids, ii) modification of gastric and intestinal motility patterns, and iii) alterations in visceral blood and lymph flow have the most significant impact on absorption.

In this paper, we have reviewed the physiological changes that occur in the postprandial gastrointestinal tract of adults. Absorption of compounds occurs mainly in the small intestine and as release from its matrix is a prerequisite to intestinal absorption, the compound should be bioaccessible in the small intestine. Therefore, we have confined us to the tract mouth to small intestine and not included the large intestine. This information will provide us a framework to adjust the early-developed *in vitro* digestion model for examining the effects of food on the bioaccessibility of compounds in adults.

2 Secretion of gastrointestinal juices

Throughout the gastrointestinal tract secretory glands are present that subserve two primary functions: first, digestive enzymes are secreted in most areas from the mouth to the distal end of the ileum. Second, mucous glands, from the mouth to the anus, provide mucus for lubrication and protection of all parts of the gastrointestinal tract.

Most digestive secretions are formed only in response of food in the gastrointestinal tract, and the quantity secreted in each segment of the tract is almost exactly the amount needed for proper digestion. Furthermore, in some parts of the gastrointestinal tract even the types of enzymes and other constituents are varied in accordance with the types of food present. The digestive secretions in response of food in the gastrointestinal tract and the residence time of food in the gastrointestinal tract are discussed in more detail in the next chapters 3-7.

Table 2.1. Daily volumes of fluids ingested, secreted, absorbed and excreted from the gastrointestinal tract.

Gastrointestinal juices	Volume ingested and secreted (liters)				Volume absorbed and excreted (liters)	
Refs.	[35]	[27]	[22]	[26]	[35]	[27]
Ingestion of food/liquid	2.3	2-3[2]	2			
Saliva	1	1	1[1]	1		
Gastric juices	2	1.5	2[1]	2.5		
Bile	1	1	0.6	0.5		
Pancreatic juices	2	1	1-2	1.5		
Intestinal juices	1		1		8.3	7-8
Brunner's glands		0.2		0.2		
Crypts of Lieberkühn		1.8		1.8		
Large intestine		0.2		0.2	0.9 + 0.1	1.6 + 0.1

[1]Saliva and gastric juices together amount 3-3.5 liters.
[2]Based on the daily secreted (6.5 L), absorbed (8.6-9.6 L) and excreted volumes (0.1 L), the daily ingestion of fluids was calculated to be 2 to 3 L.

Saliva is a fluid that is continuously secreted into the mouth. It helps clean the mouth and teeth. Ordinarily, just enough saliva is produced to keep the mucous membranes of the mouth and pharynx moist. When food enters the mouth, however, secretion of saliva increases. It lubricates, dissolves, and begins the breakdown of the food.

In the stomach food, saliva, and gastric juices are mixed to form a semi-solid chyme. The HCl secreted in the gastric juices kills bacteria and denaturates proteins. Enzymes in the gastric juices begin the digestion of proteins and aid the digestion of triglycerides.

Pancreatic juice is secreted most abundantly in response to the presence of semi-solid chyme in the duodenum. Pancreatic juice contains enzymes for digesting all three major types of macronutrients (protein, fat, carbohydrate). It also contains large quantities of bicarbonate ions, which play an important role in neutralising the acid semi-solid chyme emptied by the stomach into the duodenum.

The gallbladder empties its store of concentrated bile into the duodenum when fatty foods enter the duodenum. Bile acids in the bile play an important role in fat digestion and absorption. First, bile acids help to emulsify the large fat particles of the food into many minute particles that can be attacked by the lipases secreted in the pancreatic juice. And second, bile acids aid in the transport and absorption of the digested fat end products to and through the intestinal mucosal membrane. Furthermore, bile serves as a means for excretion of several waste products from the blood.

The function of the mucus secreted by Brunner's glands is to protect the duodenal wall from digestion by the gastric juice. The glands secrete mucus in a rapid response to irritating stimuli to protect the mucosa of the duodenum. Located on the entire surface of the small intestine are small pits called crypts of Lieberkühn. The intestinal secretions are formed by the epithelial cells in these crypts at a rate of about 1-1.8 L/day [22, 26, 27, 35]. The secretions are almost pure extracellular fluid and have a slightly alkaline pH in the range of 7.5 to 8.0. The fluid is rapidly reabsorbed by the villi [26]. This circulation of fluid from the crypts to the villi obviously supplies a watery vehicle for absorption of substances from the chyme as it comes in contact with the villi, which is one of the primary functions of the small intestine.

The small intestine normally absorbs each day several hundred grams of carbohydrates, 100g or more of fat, 50 to100 g of amino acids, 50 to 100 g ions, and 7 to 8 liters of water. As a result 1.5 L of chyme passes through the ileocecal valve into the large intestine each day [27, 35]. Most of the water and electrolytes are absorbed in the colon, leaving less than 100 ml of fluid to be excreted in the faeces.

Conclusions. The volume of fluids available in the gastrointestinal tract for a compound to dissolve in is dependent upon the volume of ingested fluids, secretions and water flux across the gut wall. About 2-3 liters are ingested per day, which is in accordance with the mean intake of adults in the Netherlands [36]. The ingested volume is received together with the endogenous secretions of saliva, gastric juices, pancreatic juices and bile by the first portion of the duodenum. These secretions total about 6 liters per day and are essential for the normal luminal digestion of foodstuffs. Most of these juices are secreted postprandially. In addition, the intestine secretes about 1-2 liters per day, to protect the epithelial cells with mucus and to improve the contact between the luminal content and the epithelial cells. This fluid is rapidly reabsorbed. In addition, much fluid (7-8 liters) is already absorbed in the small intestine. Only about 1.5 liters are presented to the colon daily, of which about 1.3 liters are absorbed, with the rest forming a component of the stool.

3 Saliva

Saliva is a fluid that is continuously secreted into the mouth. It helps to keep the mouth and teeth clean. Ordinarily, just enough saliva is produced to keep the mucous membranes of the mouth and pharynx moist. When food enters the mouth, however, secretion of saliva increases. It lubricates, dissolves, and starts the breakdown of nutrients. The effects of food on the composition and rate of secretion of saliva are summarised in Table 3.1.

Table 3.1. Composition of saliva in rest and stimulated conditions.

	rest							Stimulated						
Refs	[37]	[39]	[45]	[27]	[47]	[28]	[49]	[37]	[40]	[45]	[27]	[47]	[28]	[49]
Conc (mM)														
K^+	25			30	45	30	>20	18			20	20	20	13
Na^+	10			10	3	10	3	85			70-90	60	90	55-84
Ca^{2+}		2			2	2	2	1.5-2	1			1-2		2
Cl^-	10-25			15	23-31	10	16	50			50-70	28	45	23-40
HCO_3^-	10			10	3	10	<3	70			50-70	20-30	55	50
PO_4^{3-}		5			5.5	2	7	5.5-14	4			2-5		5
SCN^-					5.5		2-5					1		
Ureum							3.3		3			2.5		
Amylase		4 EU					0.3-1 g/l							
Mucin	0.3		1921					0.3		724				
pH	**6.2**-7.4			6.0-7.4	5.6			**7.4**	7.6			7.6		
Rate ml/min	0.6		0.4	0.5	0.1			1-10	1.1	0.9	2-10	6		

Saliva is secreted into the mouth by the salivary glands. Saliva contains two major types of protein secretion: 1) a serous secretion containing an α-amylase for digestion of starches, primarily secreted by the parotid glands, and 2) mucous secretion containing mucin for lubricating purposes secreted by the submandibular and sublingal glands. The daily secretion of saliva normally ranges between 800 and 1500 ml. Saliva pH can range between 6.2 and 7.4 (6.35-6.85 [35, 46]), with the higher pH exhibited upon increased secretion [37, 46]. Under basal conditions about 0.5 ml/min of saliva (mainly mucous type) is produced [27, 37, 41, 44, 45, 46]. Upon stimulation such as the presence of a sour taste or smooth objects in the mouth, saliva secretion is increased 4- to 20- fold [27, 42, 45]. The contribution of each gland to saliva varies with the flow rate: at rest 23% of saliva is produced by the parotid glands which may increase up to 50% at a stimulated flow rate [refs in 37]. Furthermore, the active reabsorption of sodium and chloride is decreased in response to a faster flow rate. In general at higher flow rates, sodium, calcium, chloride, bicarbonate, and protein concentrations (correlated with amylase activity [44]) increase whilst phosphate concentrations and mucin

[45] decrease and the potassium concentrations show little further change (see Table 3.1). Although an increase in amylase activity is in accordance with the higher contribution of the parotid gland to the whole saliva, it was not measured in the literature summarised here. The concentration of mucin is higher in young adults, 18-35y, compared to older >65y individuals [45]. In both groups, chewing stimulation decreased the mucin concentration by 50% (Table 3.1). The salivary amylase in the swallowed food may continue to act on starches in the stomach for about another hour before the stomach acids inactivate it [35].

Conclusions. Sour taste, chewing, and smooth objects in the mouth stimulate the saliva production. In rest condition, the flow rate is approximately 0.5 ml/min, which increases 3 to 4-fold upon stimulation with maximal flow rates of 10 ml/min. 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.

4 Stomach

The major function of the stomach is to temporarily store food and release it slowly into the duodenum. It processes the food to a semi-solid chyme, which enables better contact with the mucous membrane of the intestine, thereby facilitating absorption of nutrients. Gastric acidity kills bacteria and denaturates proteins. Pepsin begins the digestion of proteins and gastric lipase aids digestion of triglycerides.

4.1 Gastric secretion

In the fasted state, basal gastric acid secretion is 2-4 mmol/h (~60 ml) in men and slightly lower in women [28, 50, 51]. The parietal cells in the human stomach produce HCl at a concentration of 27-40 mM unstimulated but at a concentration of 98-102 mM under stimulated conditions [52]. The rate of secretion may increase from 1 ml/min under fasted conditions to 10-50 ml/min immediately after food ingestion [52, 53]. The secretion of acidic fluid continues a slower rate ~ 3ml/min until the stomach is emptied [9, 28, 53]. The major physiological stimulus for acid secretion is the ingestion of food, especially if the meal has a high protein content. The protein component of the meal possesses the greatest buffering capacity.

Pepsins are secreted as their inactive precursors, pepsinogens. Acid automatically cleaves inactive pepsinogen to active pesin and also provides the optimum pH for pepsin activity [51]. Pepsin self-catalyses the formation of additional pepsin from pepsinogen. Pepsin activity is almost completely abolished above pH 5 as these enzymes are denaturated at higher pH. Human pepsins are endopetidases, and hydrolyse several peptide bonds within the interior of ingeted protein molecules to form polypeptides but little free amino acid. Pepsin secretion increases in response to food ingestion [53].

Mucous cells secrete mucus. The major components of mucus are galactose, N-acetylglucosamine and fructose. Mucus protects the gastric mucosa from autodigestion by the pepsin and acid combination in the stomach. It contains bicarbonate, which serves to raise the local pH at the site of the epithelium without raising the bulk pH. This produces additional protection by inactivating pepsin.

4.2 Gastric pH

Fasting gastric pH has been well studied, and the generally accepted value is between 1.5 and 2 [9, 15, 22, 29, 53]. The gastric pH in the fasted state can fluctuate on a minute-to-minute basis over a range between pH 1 and pH 7, but in healthy, young subjects the fasting pH was <3 for 90% of the time. Eating of a meal results in a transit rise in gastric pH to pH 3-7 in spite of increased gastric acid secretion, due to the diluting and buffering effect of the ingested food components. The stomach volume in fasted state is 20-30 ml [15, 28]. After gastric emptying occurs, the gastric pH gradually declines until the fasted-state pH environment has been reestablished (usually within 1-2h after ingestion) [29]. The decline in gastric pH is a function of both the ability of the meal to stimulate gastric acid secretion and the rate at which the meal is emptied from the stomach. Similar profiles were observed in healthy elderly subjects [54]. However, the time required for gastric pH to return to pH 2 was significantly longer when compared with the younger subjects.

Table 4.1. Gastric pH in healthy subjects in preprandial and postprandial state.

Preprandial	Postprandial			
pH	Initial pH	Time to return to ≤ pH 2 (hours)	Number of subjects	Reference
	6.5	2	1	9
1.7	5.0	1.8	24	55
1.5	7	2.5	1	28
1.5	3.5	1.5	6	57
1.8	5.0	1.8	10	53

Conclusion. Fasting gastric pH is between 1.5 and 2. Upon eating a meal, the gastric pH rises temporary to pH 3-7. During gastric emptying, the gastric pH gradually declines until the fasted-state pH environment has been reestablished (usually within 1-2 h after ingestion).

4.3 Gastric emptying

Stomach emptying is controlled to a great extent by feedback signals from the duodenum. These feedback inhibitory mechanisms slow down the rate of gastric emptying when 1) too much chyme is already in the small intestine or 2) the chyme is excessively acid (<pH 3.5), contains too much unprocessed protein or fat, is hypotonic or hypertonic, or is irritating. In this context, the following three factors of food ingestion have a major effect on the rate of gastric emptying: the volume of the meal, its osmotic pressure and its chemical composition. With liquid meals, the rate of emptying is greatest when the volume is greatest, and that means that emptying is fastest at the beginning of digestion of a meal [56]. The fast initial gastric emptying after ingestion of a meal 10-40 ml/min [ref in 22, 28, 56] is followed by a more slow emptying rate of 2-4 ml/min [9, 28, 53]. Low caloric fluids empty from the stomach according to first order kinetics with $T_{1/2}$ ranging from 10-60 min [5, 12, 18, 28, 30, 57]. Consequently, the greatest bulk of gastric content of a liquid meal is delivered to the duodenum before much gastric acidification has occurred.

Composition of the food is important for gastric emptying. The rates of emptying of the three major foodstuffs in liquid form are regulated so that equal numbers of calories are delivered to the duodenum in the same time (2 kcal/min [38, ref 44 in 22]. At a calorie density of 1 kcal/ml, about 2-2.5 ml/min are emptied, whereas at 0.2 kcal/ml, about 10 ml/min are emptied [ref 44 in 22]. There are roughly 9 kcal in a gram fat and 4 kcal in a gram of carbohydrate or protein. Consequently, an emulsion of 4 g of fat is emptied in the same time as a solution of 9 g of carbohydrate or protein. Because fat contains the most calories per gram, meals composed largely of fat of any kind may remain in the stomach 20 h [28]. Furthermore, it was shown that gastric concentrations of fat and protein may behave in a different way due to separation of the liquid test meal in the stomach into two phases, one lower, containing water, sugar, and PEG, and one upper where fat was concentrated [9]. This resulted in a much faster emptying of sugar $T_{1/2}$ was 52 min than for fat $T_{1/2}$ was 154 min. Furthermore, the digestion products of fat, carbohydrate and protein are the compounds that affect the receptors in the duodenum, which in turn control the rate of emptying.

The last factor affecting gastric emptying is the osmotic pressure of the meal. The duodenum adjusts the osmotic pressure of its content to isotonicity. This was also found for the osmolar concentration in the jejunum, which was rather constant around 300 mosm/l despite ingestion of a liquid test meal of 940 mosm/l [9]. In accomplishing isotonicity, gastric emptying is

regulated by the osmotic pressure of duodenal contents. Therefore, a meal with a higher osmotic pressure will be emptied slower.

Table 4.2 Gastric emptying of liquid and solid phases in the fed state.

Food			Gastric emptying			Ref.
Kcal	Liquid/solid	Determined by	T½ (min)	kinetics	Complete empty (min)	
610	Solid	Food component		linear	285	6
~286	Liquid	Glucose/PEG Fat	52 154	exponential		9
530	Solid Liquid	Radioactive markers	114 (men) 160 (women) ~ 60	Linear Linear exponential		12
492 661 930	Liquid	$^{51}CrCl_3$	138 156 222	exponential		13
196 621 1920 196 621 1920	Solid Liquid	Colloid-labeled marker Radioactive marker	77 145 277 40 81 178	Linear exponential		58
208	Solid solid		130 70-117			Refs in 30
400 900		Scintigraphy, 0.7-1.2 mm pellets	119 255			Ref in 30
	Semi-Solid (mashed potato meal)	Tomography and scintigraphy	60	linear	120 min	57

Much information on gastric emptying in the fed state is obtained by gamma-scintigraphy of small pellets or tablets [3, 5, 16, ref 57 in 22] or radio-labeled food [6, 58], or by gastrointestinal tubes in the duodenum and/ or stomach [9, 12, 13, 58]. Half-emptying times (or complete emptying) for solid and liquid meals are summarised in Table 4.2. Solid meals (and nutrient fluids) empty from the stomach according to zero order kinetics, with emptying dependent on caloric concentration, as was described above for liquid meals. In the distal stomach the particle size of ingested food is reduced to a fluid-like consistency. The size of the food particles, which are emptied into the duodenum, is mostly < 2 mm. Because the meal emptying depends on the calorie content, the size of the meal is very important

Consequences for the bioaccessibility of compounds. Generally, delayed gastric emptying has the potential to increase absorption of poorly water-soluble compounds by increasing the time

available for dissolution. For acid-labile and ionizable compounds, food increases the residence time in the acidic environment, which impacts on solubility and stability issues. However, the increase in stomach pH in response to food ingestion makes it difficult to predict the likely overall effect on bioaccessibility. Following a liquid meal, much of the meal is released in the duodenum before acidification of the stomach occurs. This would have consequences for compounds that are released from their matrix at low pH.

Conclusions. The gastric emptying is determined by three major factors: the volume of the meal, its osmotic pressure and its the caloric content of the food. The rates of emptying of the three major foodstuffs (fat, carbohydrate and protein) are regulated so that equal numbers of calories are delivered to the duodenum in the same time (2 kcal/min). Fluid meal generally empty from the stomach according to first order kinetics with $T_{1/2}$ ranging from 10-60 min. Solid meals empty from the stomach according to zero order kinetics, with emptying dependent on caloric concentration ($T_{1/2}$ ranging from 60 – 277 min).

5 Small intestine

5.1 Secretion of bile

Gallbladder volume, emptying, and refilling was measured by real-time ultrasonography after ingestion of a standard breakfast (40% fat) in 12 healthy men and women. Gallbladder emptying was biphasic: 50% emptying the first 30 min after ingestion of the meal followed by a 3-fold slower emptying. The gallbladder volume remained small until refilling began 4h after ingestion of the food when gastric emptying neared completion (~13% remained) [6]. In another study in 18 healthy volunteers, the gallbladder volume was reduced by 23% and 59% after ingestion of medium chain triglyceride and long chain triglyceride meals, respectively [21].

The bile concentration in the distal duodenum or jejunum was determined using intestinal perfusion techniques. Fasting bile concentrations were in the range 1.5-5 mM in the proximal small intestine (Table 5.1). Although fasting bile concentrations were determined after an overnight fast, the stomach was not always completely empty [13], which might result in higher fasting bile concentrations. Bile concentrations vary widely between individuals but average values are similar in the duodenum and jejunum. Levels fall rapidly in the ileum where bile salts are absorbed by an active transport mechanism. Although very low, the bile salt concentration delivered to the colon may significant increase after ingestion of a meal [10].

After eating, the bile output and luminal concentrations were highest in the duodenum 30 min after ingestion of the meal (contraction of gallbladder), followed by a lower rather constant bile concentration, which declined when the stomach was almost empty [13]. Total daily output increased with the calorie content of the test meal, which was related to the emptying of the stomach [13]. Comparing the effects of ingesting medium chain triglyceride and long chain triglyceride meals, the jejunal bile acid concentrations increased significantly more after the ingestion of long chain triglyceride [21]. However, in another study using medium chain triglycerides as the main source of fat content [13], bile acid secretion increased within 60 min after ingestion of the meal. Overall, mean bile concentrations in the proximal small intestine vary between 7 and 15 mM after eating as summarised in Table 5.1.

Conclusions. The gallbladder contracts in a reaction to fat entering the duodenum. This results in a peak concentration of bile in the duodenum followed by a lower “constant” bile concentration. Bile, mostly produced by the liver, is secreted in the duodenum as long as there is fat in the duodenum. The gallbladder only starts refilling with bile when the stomach is almost empty [6]. The amount of bile secreted by the gallbladder depends on the amount and the type of fat in the duodenum. In the proximal small intestine, fasting bile concentrations were in the range 1.5-5 mM and increased to 7-15 mM after eating. The bile concentration was elevated until the food was emptied from the stomach.

Table 5.1 Bile concentrations in the distal duodenum and proximal jejunum.

Preprandial state				Postprandial state			
Bile[1,4] (mM)	Bile measured	Flow[2] (ml/min)	Ref	Bile[4] (mM)	Bile measured	Flow (ml/min)	Ref
1.6 (d)	11-13 µmol/min	6.9	10	10.6 (d)	120 µmol/min	11.3	10
5.2±2.3 (d)			20	14.5 (5.2-39.6) (d)			20
2.7-4 (d)	7-10 µmol/kg/h	2	19	8-21 (d)	20.8-54.9 µmol/kg/h	3	19
1.7 (d) 3.3 (d)	3 9[3] µmol/kg/h	2 3	13	7 (d)	18 µmol/kg/h	3	13
4 (j)			21	15.2[5] (j) 5.0[5] (j)			21
				12 (d) 8.8 (j)	33 µmol/min	3.75	7

[1] To calculate the bile concentration, it was assumed that the mean body weight was 70 kg. Then the bile concentration (in mM) was calculated by dividing the bile flow (in µmol/min) over the jejunal flow rate (ml/min).
[2] The jejunal flow rate was assumed to be 1 ml/min in the fasted state and 3 ml/min in fed state, respectively [22]. A higher flow rate has been used to calculate the bile concentration when the perfusion rate was higher than the above mentioned flow rates. The flow rates were measured in reference [10].
[3] Stomach was not completely empty.
[4]Bile concentration was determined in distal duodenum (d) or in jejunum (j)
[5] Jejunal bile concentration after ingestion of long chain triglyceride and medium chain triglyceride meals were 15.2 mM and 5.0 mM, respectively.

5.2 Secretion of pancreatic fluids

Pancreatic juice is secreted most abundantly in response to the presence of semi-solid chyme in the duodenum. Pancreatic juice contains enzymes for digesting all three major types of food. It also contains large quantities of bicarbonate ions, which play an important role in neutralising the acid semi-solid chyme emptied by the stomach into the duodenum. The exocrine secretions of the pancreas consist of a high-volume aqueous secretion containing bicarbonate and a low-volume aqueous secretion containing digestive enzymes. The basal flow rate of bicarbonate secretion typically increased from 0.3 ml/min to 4.0 ml/min during stimulation [ref 165 in 8]. When the flow rate was at a lower value of 0.6 ml/min, the concentration of bicarbonate was ~25 mEq/L, resulting in a secretion rate of ~1 mEq/h. [ref 166 in 8]. The pH of the alkaline fluid entering the duodenum at high output rates is ~8 [ref 192 in 8]. The secretion of bicarbonate is largely regulated by the amount of acid entering the duodenum. The activity of lipase is 7 to 10-fold higher than the activity of amylase [7, 9], but the concentrations of all digestive enzymes in the duodenum increased 2.5 to 5-fold in response to stimulation/eating [7, 10, 13]. The concentration of the enzymes in the duodenum was independent of the caloric intake [10], but increased with caloric content when the calories were infused directly into the duodenum [7]. This supports the theory that equal numbers of calories are emptied from the stomach into the duodenum (Chapter 4).

Conclusions. Pancreatic juices are secreted in response to the presence of semi-solid chyme in the duodenum. Bicarbonate is secretion to neutralise the amount of acid entering the

duodenum, whereas the enzymes start the digestion of all three major types of food. The enzyme concentration increased 2.5 to 5-fold in the duodenum in the response to food components.

5.3 Transit time

The small intestinal transit times of various studies are shown in table 5.2. In general, there is little difference in the mean transit times through the small intestine between the fed and fasted states. Malgelada *et al.* [59] showed that the intestinal residence profiles for liquid and solid components of a meal were not significantly different, and that the transit time was in the order of one to three hours. The Nottingham Group has undertaken various transit studies on a large number of volunteers. The overall mean small intestinal transit time was of the order of 3-4 hours with a standard deviation of 1 hour [57].

Various techniques have been used to determine the gastrointestinal transit of foodstuffs and more particularly, pharmaceutical dosage forms. In early studies, radiography was used. A series of X-rays was taken of a radiolabeled dosage, for example by inclusion of barium sulphate, in order to monitor the transit of the dosage form through the different regions of the small intestine. The hydrogen breath test relies upon the metabolism of certain carbohydrate materials, for example lactulose, by the microbial flora within the caecum and large intestine. Osmotic effects of the administered material and bacterial overflow in the terminal ileum may cause underestimation of transit times. By using intubation-perfusion techniques, the small intestinal content can be aspirated at different locations in the small intestine. In this manner the transit time between aspiration points, with a well-known distance from each other, can be calculated. The transit time may be affected by a high perfusion rate. In the γ-scintigraphic method, the dosage from is labelled with a small amount of suitable gamma emitting radionuclide (technetium-99m or indium-111) and the transit of the material followed using a conventional gamma camera. The presence of the dosage form in the stomach and its gastric emptying can be well defined and its arrival in the ileocaecal region is also easy to assess. It is normal practice to take the time for 50% gastric emptying and the time for 50% colonic filling and to use the difference between these two numbers as a time for small intestinal transit. Small intestinal transit times determined by these techniques in human subjects are summarised in table 5.2.

Flow of intestinal content in the fasted state is largely intermittent with peaks of flow associated with passage of an activity front through the segment. Values stay largely between 0 and 2 ml/min, with an average of 0.7 ml/min in the jejunum and 0.43 ml/min in the ileum [ref 46 in 22]. After eating, flow rates increase to average of about 3 ml/min and 2 ml/min, respectively, with values ranging from 0-7 ml/min [ref 46 in 22]. A similar jejunal flow rate of 3 ml/min after ingestion of a liquid meal [9]. In another study [21], the postprandial ileal flow was 0.8 ml/min and 2 ml/min after ingestion of medium chain triglyceride and long chain triglyceride meals, respectively.

Table 5.2. Small intestinal transit times.

Transit time (min)		Technique		Ref
Fasted	fed			
241 267	245 265	light density pellets high density pellets	γ-scintigraphy	3
240[1] 198[1]		liquid marker tablet	γ-scintigraphy	5, 18
	74(women) 38 (men)	Lactulose	H_2 excretion	6
	209 182	Pellet Tablet	γ-scintigraphy	16
	60	phenol red	perfused intubation	21
	180	radiolabeled foodstuff		59
	68-136[2] 73 126 156-228	PEG 400 Barium sulphate Pellets	perfused intubation H_2 excretion X-ray γ-scintigraphy	57

[1]In the presence of high concentrations of mannitol or sodium acid pyrophosphate but not in the presence of sucrose, the small intestinal transit was significantly reduced.

[2] For calculation of the small intestinal transit time, it was assumed that the length of jejunum is 2.4 m and length of ileum is 3.6 m [57 pg 49]. Assuming that the jejunum is about 1 m long and the ileum measures about 2 m [35], the small intestinal transit time is ~70 min.

Conclusions. There is little difference in the mean transit times through the small intestine between the fed and fasted states. Mean small intestinal transit time is of the order of 3 hours (range 1 to 6 hours). The flow of intestinal content increases ~ 3-fold after ingestion of food.

5.4 Small intestinal pH

Reported regional intestinal pH values in healthy human subjects in preprandial and postprandial states are compiled in Table 5.3. The intestinal pH values are considerably higher than gastric pH values due to the neutralization of incoming acid with bicarbonate secreted by the pancreas. Furthermore, the pH values increase gradually between the duodenum and ileum. In the postprandial state, the pH in the duodenum is lower than that in the fasted state. No major differences in pH values were found in the jejunum and the ileum upon meal ingestion.

Table 5.3. pH in the small intestine in healthy humans in the preprandial and postprandial state. [refs in 22, and in 11].

Intestinal site	Preprandial pH	Postprandial pH
Duodenum (mid-distal)	4.9	5.2
	6.1	5.4
	6.3	5.1
	6.4	
Jejunum	4.4 – 6.5	5.2 – 6.0
	6.6	6.2
Ileum	6.5	6.8 – 7.8
	6.8 – 8.0	6.8 – 8.0
	7.4	7.5

Conclusions. The pH values in the small intestine gradually increase between duodenum and ileum, from pH 5.5 to 7.5. In the duodenum, the pH after eating is lower 5-5.5 than compared to the fasted state pH 6. However, in the jejunum no major differences in pH 6-6.5 were found upon ingestion of a meal.

6 Food

6.1 Food intake for a hot meal by adults in the Netherlands

Mean intake of energy and macronutrients per hot meal in men and women in the Netherlands are based on the food consumption measurements (VCP) [36]. The mean data in Table 2 pages 52-73 of the VCP were used to calculate the overall means without using a weighing factor. Based on the individual intake of a hot meal, the median and the 5 and 95 percentile were calculated 736 kcal, 299 kcal and 1486 kcal, respectively. These values can be used to estimate a hot meal with low caloric intake, which will have a short residence time in the stomach, and a hot meal with high caloric intake, which will have a long residence time in the stomach (Table 4.2). These values will be used to estimate the amount of food to be used in the in vtiro digestion model to simulate a low, normal and high food intake.

Table 6.1. Mean intake of energy and nutrients per hot meal in men and women aged 19-65 in the Netherlands.

	Intake hot meal	Intake hot meal per 100g
	Mean	mean
Kcal	816	106
Protein (g)	39	5.1
Carbohydrate (g)	75	9.8
Fat (g)	39	5.0
Water (g)	604	78.8
Fiber (g)	9	1.2
Total weight (g)	766	100

6.2 Digestion of macronutrients

Carbohydrate digestion. In the mouth, salivary amylase converts starch (polysaccharide) into smaller fragments of starch. Salivary amylase may continue in the stomach for a while, as long as the pH is high, so that up to 35% to 48% of starch can be broken down into smaller fragments in the stomach [28]. The acidic pH of the stomach destroys salivary amylase and blocks its activity. Thus few starches are reduced to maltose by the time chyme leaves the stomach. Those starches not already broken down into di- or trisaccharides or α-dextrins (5-10 glucose units) are cleaved by pancreatic amylase. Amylase acts on both glycogen and starches but not on cellulose. Brush border enzymes digest the di-, trisaccharides, and the α-dextrins into monosaccharides, which can be absorbed.

Protein digestion. In the stomach, pepsin converts proteins to peptides (small fragments of proteins). When radioiodinated human serum albumin (RISA) was added to a liquid meal taken by normal men, samples recovered by tube from the region of the pylorus contained 85-90% of the administered 131-I in a form precipitable by 5% phosphotungstic acid. This means that the largest part of protein digestion occurs beyond the stomach [28]. Enzymes in pancreatic juice, trypsin, chymotrypsin, carboxypeptidase and elastase, continue to break down proteins into peptides. Protein digestion is completed by the peptidases in the brush border (aminopeptidase and dipeptidase).

Lipid digestion. Lipid digestion is the result of three sequential processes involving i) dispersion of fat globules into a coarse emulsion of high surface area, ii) enzymatic hydrolysis of the fatty acid glyceryl esters (primarily triglyceride lipid), and iii) dispersion of

the products of lipid digestion into an absorbable form. In an adult, most lipid digestion occurs in the small intestine, although some occurs in the stomach through the action of lingual and gastric lipases.

i) Lingual lipase secreted by the salivary gland and gastric lipase secreted by the gastric mucosa are responsible for initiating hydrolysis of ingested triglyceride to the corresponding diglyceride and fatty acid within the stomach. Liberation of these amphiphilic lipid digestion products, in combination with the shear produced by antral contraction and gastric emptying, facilitates formation of a crude emulsion that empties into the duodenum.

ii) The presence of lipid in the duodenum stimulates secretion of bile salts, biliary lipids, and pancreatic juice. Biliary lipids adsorb to the surface of the crude emulsion, stabilising it and further reducing droplet size to about 1 mm. Subsequently, the majority of triglyceride hydrolysis occurs under the action of pancreatic lipase, which requires the presence of colipase to be active in the presence of bile salts. It is an interfacial enzyme that acts at the surface of the emulsified triglyceride droplets to quantitatively produce the corresponding 2-monoglyceride and two fatty acids. These digestion products are effective emulsifying agents, and because fatty acid promotes binding of the lipase/colipase complex to the emulsion surface, the lipolytic process is essentially selfpromoting.

iii) Phospholipids are hydrolysed to the corresponding lysophospholipids by phospholipase A_2 prior to absorption, and in quantitative terms, the majority of phospholipid reaches the gastrointestinal tract via biliary secretion with only modest inputs from dietary sources.

Conclusions. Although the chyme entering the small intestine contains partially digested carbohydrates, proteins and lipids, the completion of the digestion of carbohydrates, proteins and lipids is a collective effort of pancreatic juice, bile, and intestinal juice in the small intestine.

6.3 Absorption of nutrients

Digestion of food from the mouth through the small intestine is directed toward changing food into forms that can pass through the epithelial cells lining the mucosa into the underlying blood and lymphatic vessels. These forms are monosaccharides (glucose, fructose, and galactose) from carbohydrates; single amino acids, dipeptides and tripeptides from proteins; and fatty acids, glycerol, and monoglycerides from triglycerides. Facilitated diffusion or active transport mediates absorption of monosaccharides. Most proteins are absorbed as amino acids or di/tripeptides by active transport processes that occur mainly in the duodenum and jejunum. About half of the absorbed amino acids are present in food, whereas the other half comes from proteins in digestive juices and dead cells that slough off the mucosal surface. Although the specific absorption mechanisms have not been elucidated, the common role of mixed micellar phase in solubilising poorly water-soluble lipid digestion products and providing a concentration gradient for absorption of monomeric lipids is generally accepted [see refs in 8]. Micelles are not absorbed intact and lipids are thought to be absorbed from a monomolecular intermicellar phase. In support of the importance of the intermicellar phase, concentrations of cholesterol several times higher than the saturated aqueous solubility can be achieved in the intermicellar environment. The dissociation of monomolecular lipid from the mixed micellar phase prior to absorption may be stimulated by a microclimate of lower pH associated with the absorptive site. Although evidence is

mounting to suggest that uptake of lipid digestion products may not be entirely via simple diffusion, further work is required to clearly define the factors and processes involved.

About 90% of all absorption of nutrients takes place in the small intestine. The other 10% occurs in the stomach and large intestine. Any undigested or unabsorbed material left in the small intestine passes on to the large intestine. By infusion of proportions of protein, fat, and carbohydrate directly into the duodenum, it was found that fat is more rapidly absorbed than carbohydrate or protein [7]. Within the first 60 cm of the small intestine (mean transit time 31 min), about 80% of protein and more than 90% of carbohydrate and fat was absorbed. In another study, the absorption of sugar, fat, and protein were determined during passage of upper 75 cm of the small intestine in a healthy volunteer [9]. Less fat (53.1%) was absorbed than sugar (81.5%) in the upper 75 cm of the small intestine during 4 hours after consumption of the liquid meal. The lower fraction of fat absorbed during this period was mostly due to the delayed gastric emptying of fat compared to sugar.

Conclusions. Approximately 90% of all absorption of nutrients occurs in the duodenum and jejunum.

6.4 Impact of differences in pre- and postprandial gastrointestinal state on bioaccessibility of compounds.

Although physical and chemical interactions may occur between compounds and specific food components, altered postprandial absorption is generally a function of the changes associated with conversionfrom the fasted to the fed state. Changes due to to i) secretion of gastric acid and bile and pancreatic fluids, ii) modification of gastric and intestinal motility patterns, and iii) alterations in visceral blood and lymph flow have the most significant impact on absorption.

The pH differences in the contents of the upper gastrointesinal tract between fed and fasted states can influence the dissolution and absorption of weakly and acidic and basic compounds. The dissolution rate of weak bases is typically greater in gastric fluids than in the intestine in the fasted state, whereas that of weak acids is at a minimum in the stomach and increases as undissolved compound is transported to the less acidic regions of the intestine. Therefore, elevation of gastric pH following a meal may enhance the dissolution of a weak acid in the stomach but inhibit that of a weak base. Furthermore, because food inhibits the rate of gastric emptying, prolonged retention in the stomach may increase the proportion of compound that dissolves prior to passage into the small intestine. The primary site of drug absorption is usually the small intestine because of its greater mucosal surface area and range of transport mechanisms, so the class of compounds most vulnerable to pH-related changes in post-prandial absorption are poorly water-soluble weak bases.

Lipids are a major constituent of food that can inluence the absorption of compounds. Ingested lipid decreases gastric motility, and the presence of lipid digestion products within the upper small intestine induces secretion of biliary and pancreatic fluids that dramatically alter the luminal environment. The presence of bile may improve the bioavailability of poorly water soluble compounds by enhancing the rate of dissolution and/ or solubility. An increase in the rate of dissolution can occur by an effective increase in surface area (enhanced wetting) or an increase n solubility via micellar solubilisation. Hasegawa *et al.* [ref 140 in 8], studied the effect of food on the bioavailability of nicotinate prodrug of vitamin E (alpha-tocopheryl

nicotinate) in humans. The oral bioavailability was 28-fold increased due to improved dissolution and solubilisation. Oral bioavailability of etrinate increased 3-5 fold when administered with a high fat meal or milk compared with fasted administration (Refs 146 and 147 in 8]. The amount of parent compound increased whereas the metabolism of etrinate seemed to be reduced. Another factor affecting the postprandial absorption of highly lipophilic compounds is the stimulation of lymphatic transport. The rate of fluid transport in the intestinal lymphatics is ~0.2% (v/v) compared with portal blood [ref 132 in 8]. Consequently, the major factors for lymphatic transport to be significant contributor to bioavailability include coadministraiton of a lipid source, metabolic stability, and sufficiently high lipophilicity. In terms of a “ball mark” estimate, the log P of the compound needs to be >5, with a triglyceride solubility of at least 50 mg/ml, before lymphatic transport is likely to be a major contributor to bioavailability [refs 116 and 133 in 8]. Examples of compounds that are transported (to varying degrees) by the intestinal lymphatics include lipophilic vitamins and vitamin derivatives, xenobiotics such as DDT and related analogues, benzo-[a]-pyrene, and numerous lipophilic prodrugs [Refs 25, 118, 127, 128, 129, 130 in 8].

7 Proposal for *In vitro* digestion model to mimic the fasted and fed state in humans.

The *in vitro* digestion model, developed in our laboratory, is based on the model described by Rotard *et al.* [33]. This model simulates the physiological conditions of the gastrointestinal tract from mouth to the small intestine in the fasted state. As described in the previous chapters, ingestion of food greatly affects the physiological conditions in the gastrointestinal tract. Therefore, the *in vitro* digestion model will have to undergo some alterations to simulate the fed state of the gastrointestinal tract. Possible changes compared to the present *in vitro* digestion model and compared to the recipes described by Rotard *et al.* [33] are indicated in chapters 7.1 and 7.2.

7.1 Volumes of ingested and secreted fluids

The volume of fluids available in the gastrointestinal tract for a compound to dissolve in is dependent upon the volume of ingested fluids, secretions and water flux across the gut wall. About 2-3 liters are ingested per day, which is in accordance with the mean intake (2.4 liters) by adults in the Netherlands [36]. The ingested volume is received together with the endogenous secretions of saliva, gastric juices, pancreatic juices and bile by the first portion of the duodenum. These endogenous secretions total about 6 liters per day and are essential for the normal luminal digestion of foodstuffs. Most of these juices are secreted postprandially. In addition, the small intestine secretes about 1-2 liters per day, to protect the epithelial cells with mucus and to improve the contact between the luminal content and the epithelial cells. The volume of the small intestinal secretions was taken into account in the *in vitro* digestion model developed for ingestion of soil by children. However, the composition of the pancreatic/small intestinal juice did not change from the pancreatic juice. As the fluid secreted by the small intestine is rapidly reabsorbed [27] and already much fluid is absorbed in the small intestine (Table 2.1, chapter 2), it was now discussed not to take these volumes (fully) into account for the total volume available for a compound to be released in from its matrix. It is, therefore, proposed to use the daily-secreted volumes described by Tortora [35] as the basis for the *in vitro* digestion models in project V/630030. The same ratio of secreted fluids has been used by Rotard *et al.* [33].

Table7.1. Comparison of the volumes (to be) used in the in vitro digestion model with the daily volumes of fluids ingested and secreted from the gastrointestinal tract according to Tortora.

Compartment Volume in liters	Tortora [35]	Rotard [33]	Fasted Child "soil"model [34]	*Fasted Adult*[1]	*Fed Adult*[1]
Ingestion of food/liquid	2.3				*1.5 (1-2)*
Saliva	1	1	1	*1*	*1*
Gastric juices	2	2	1.5	*2*	*2*
Bile	1	1	1	*1*	*1 (0.5-1)*
Pancreatic juice	2	2	3	*2*	*2*

[1] Proposal for ratio of the volumes in each compartment to be used in project V/630030.

Although the daily mean volume of food and liquids ingested, as determined by water content, is 2.4 liters for adult man and women (19-65 years), half of the daily volume intake is consumed in between meals (1.2 liter) [36]. On the other hand, most of the gastrointestinal digestion juices are secreted in reaction to food consumption. As the water content in the hot meal is ~80%, a total weight of food ingested per day can be calculated of 1.2 liters x 100%/80% is 1.5 kg (liters). We propose, therefore, to use the following weight (volume) ratios: food/saliva/gastric juice/ bile/pancreatic juice is 1.5/1/2/1/2 (see Table 7.1). To apply in the *in vitro* digestion model, the unit is 6 g (cq 6 ml) so that it is estimated that a double amount of food can be used in our system (max of 60 ml).

7.2 Proposed alterations of the *in vitro* digestion model as result of food

For fasted conditions in adults the ration of secreted fluids is according to Tortora and Rotard (Table 7.1). The major changes in composition of the fluids, volume ratio, and incubation time of the various steps in the *in vitro* digestion model as result of food are shown in Table 7.2. These changes are based on the physiological data reviewed in Chapters 2-6. Unless otherwise stated, it is assumed that the concentration of ions do not change from the fasted to fed state. The changes are shorlty discussed below and are compared to other *in vitro* digestion models.

Table 7.2 Proposal for changes of the in vitro digestion model as result of food ingestion.

Digestion phase	Composition	Volume	Incubation time
Food	Standard: protein/carbo/fat=1/2/1 (w/w/w)	1.5	
Saliva	Amylase 2x↑, Na^+↑, Cl^-↑, HCO_3^-↑, mucin↓, pH↑ (lipase)	1	0-5 min
Stomach	Pepsin 2-3x↑ pH 5 (3-7) →(60-120 min)→ pH2	2	<300 kcal: 2h 450-700 kcal: 4-7h >900 kcal: 6-20h
Bile	Standard meal: 8 mM Fat meal: 15 mM	1	2h
Pancreas	Enzymes 2-3x↑, bicarbonate↑ (mucin)	2	

Food. On average, the adult population in the Netherlands consumes equal amounts of protein and fat and a double amount of carbohydrates when eating a warm (evening) meal (see Chapter 6.1, Table 6.1). By reducing the intake (reducing the ratio of volume of food) a low caloric meal can be obtained. A high caloric meal can be obtained by doubling the amount of food intake or by addition of extra oil to the standard meal. We thought of using Olvarit infant formulas as a standard meal since the food is ready for use and natural products such as meat, vegetables and potatoes/rice/pasta are the source for protein, carbohydrate and fat contents. A selection of Olvarit infant formula products is shown in table 7.3. The products were selected based on the caloric content and composition of the macronutrients. All the Olvarit infant formula products are low in energy content: 280 kJ-359 kJ compared to the mean VCP intake of 445 kJ/100 g. Product 451 has a composition of macronutrients (protein/carbohydrate/fat), which is comparbale to the mean consumption. However, the energy content is only 63% of the mean intake. The other food products have relatively too many carbohydrates and far too little fat compared to our consumption profile. By addition of 2-2.5 g oil (fat) the products 404, 452, 455 and 282 are comparable: the energy content is

within 5-105 of the mean VCP intake, however, the relative carbohydrate content remains high (carbohydrate: protein = 2.5-2.9 instead of 1.9). Product 224 has the right proportion of the macronutrients, after 1 g fat is added, but 15% less energy content than the mean VCP intake.

Table 7.3. Composition of macronutrients in several Olvarit infant formulas (analysis per 100g food).

Food (product number)	kJ	Protein Gram	Carbohydrate Gram	Fat Gram	Remark
Mean intake VCP	**445**	**5.1**	**9.8**	**5.0**	**1.2 g fiber**
451	280	3.0	7.0	3.0	
404	340 434	4.0 4.0	11.5 11.5	2.0 4.5	 Addition of 2.5 g fat
224	350 388	4.5 4.5	9.0 9.0	3.5 4.5	 Addition of 1 g fat
452	350 425	4.0 4.0	11.0 11.0	2.5 4.5	 Addition of 2 g fat
455	330 424	4.4 4.4	11.0 11.0	2.0 4.5	Too much fiber? Addition of 2.5 g fat
282	359 434	4.1 4.1	11.4 11.4	2.6 4.6	 Addition of 2 g fat

The Olvarit infant formulas (> 15 months) contain 3 to 4.5 mg protein / 100g. This is lower than the mean intake of protein by adults during the hot meal (5.1 g protein/100g, Table 7.3). Based on the protein content in Olvarit food products, a protein concentration of 10-15 mg / ml stomach content is calculated ((1.5/4.5)x(3-4g/100g)). This protein concentration per total volume in the stomach is rather low but within the range compared to in vivo studies: 10-15 mg protein / ml (9 g protein / 300 ml food + 300-600 ml gastric secretions) [9], 37-50 mg protein/ml (33.3 g protein / 300 ml food + 300-600 ml gastric secretions) [12], 28 mg/ml (16 g protein / 540 ml gastric emptying) [21].

Compared to other *in vitro* digestion models, the protein content per total gastric volume was 38 mg protein / ml (4 g protein / 10.5 ml) [31]. Based on dry matter / gastric volume 58 mg /ml (7 g dry matter /120 ml) [32A], and 200 mg/ml (60 g dry matter / 300 ml) [Minekus chapter 4] compared to our proposal 47 mg/ml (14 g dry matter / 100 g food x 1.5/4.5). In conclusion, the protein and dry matter content is, although low, in a similar range as other *in vitro* digestion models use.

Saliva. The composition of saliva under stimulated conditions can be changed as was proposed by Marieke Hofhuis. The concentrations of potassium, phosphate and thiocyanate can be kept the same. Although I have thus far no numbers for the change in amylase activity when a stimulated saliva is produced, several papers mention that amylase is increased,

which is in accordance with the doubled contribution of the parotid glands to the whole saliva (Chapter 3). The concentration of mucin may be reduced compared to resting conditions [45]. The saliva pH under stimulated conditions is slightly increased compared to resting conditions (Chapter 3). Therefore, the saliva pH may be increased to pH 6.8 ± 0.2. Thus, the composition of saliva to simulate stimulated conditions is proposed to change as follows: Na^+ (57 mM), Cl^- (37 mM), and HCO_3^- (20 mM), amylase (290 mg/1000 ml), mucin 25 mg / 1000 ml), and pH 6.8 ± 0.2.

Alternatively, lipase may be added to the saliva to start the digestion of lipids (see chapter 3) and the incubation time with saliva may be reduced to 0 min. In the time all the tubes are filled with saliva, the tubes have incubated for a few minutes with saliva. As the pH in the stomach in the fed state is much higher than in the fasted state, digestion of carbohydrates by amylase may continue in the stomach until the pH is lowered to a pH of 2. For practical reasons, all the tubes can be filled with saliva and immediately after that add the first portion of gastric juice.

Stomach. Pepsin secretion increases in response to food ingestion [53]. Several other *in vitro* digestion models use 1 mg/ml [32], 1.25 mg/ml [24, 31], 3 mg/ml [30], whereas we use 1 mg pepsin/ml gastric juice, which is subsequently diluted by saliva resulting in 0.67 mg pepsin/ml. The concentration of pepsin will be even further diluted by addition of food. Therefore, the concentration of pepsin in the gastric juice should be 3-fold increased.

The initial high stomach pH after eating is caused by dilution of the gastric volume by the food and saliva and by the H^+ binding capacity of the food. The gastric volume under fasted conditions is only 20-30 ml [15, 28], but gastric secretions are increased in response to food. This can be simulated by an initial addition of a small volume of gastric juice, for example 0.5 unit (3 ml), followed by the rest of the gastric juice (1.5 unit, 9 ml) after 60-120 min. In this way, the initial high stomach pH (pH 5 ± 2) followed by a lower stomach pH (pH 2) may also be simulated.

Bile. In the proximal small intestine, bile concentrations under fasting conditions were in the range of 1.5-5 mM and increased to 7-15 mM after eating (Chapter 5, Table 5.1). The bile concentration was elevated until the food was emptied from the stomach. The secretion of bile is dependent on the amount and type of fat (Chapter 5.1). The concentration of bile in the "soil"digestion model is 1.7 mM (1.8 g bile = 3.6 mmol/300 ml x 9 ml/58.5 ml), which is within the range of bile concentration in the fasted state (Chapter 5.1). It is proposed to increase the concentration of bile to 8 mM in the chyme after eating a standard meal and to 15 mM after eating a fat meal. Thus, the concentrations of bile in the bile solutions should be 60 mM (8x1 vol/ 7.5 vol) and 112.5 mM, respectively. The bovine bile is a mixture of glycine and taurine conjugated salts and, therefore, a mean molecular weight of bile salts of 500 can be calculated. This results in weighing 3 g and 5.6 g bile / 100 ml bile solution to obtain 8 mM and 15 mM bile in the chyme, respectively. By proceeding this way, the actual bile salt concentration in the chyme will be lower since the exact bile salt concentration in the commercial bile extract is unknown but is garantueed to be higher than 55% according to the product sheet of Sigma. In other *in vitro* digestion models the concentration of bile mimicking the fed state was 10 mM in jejunum [2], 5.8 - 7.8 mM [32A en 32B], 4 mM [31], 3 mM [30], 1 mM (fasting conditions) [33], 2.8 mM [24].

Pancreatic juice. Pancreatic juices are secreted in response to the presence of semi-solid chyme in the duodenum. The amount of bicarbonate secreted is as much as is needed to

neutralise the amount of acid entering the duodenum. The concentration of bicarbonate is likely to be increased but the amount depends on the H^+ binding capacity of the gastric content that is emptied in the duodenum (thus likely to depend on the type of food and the amount of food).

The concentrations of digestive enzymes increased 2.5 to 5-fold in the duodenum in the response to food components (Chapter 5.2). In the pancreatic secretions, the activity of lipase is 6 to 10-fold higher than the activity of amylase [7,9,10,13]. In the porcine pancreatin, which is added in the digestion model, the activity of amylase is higher than the activity of lipase. Humans with pancreas insufficiency (not included insulin) are supplied with pancreatine pellets from porcine origin [61]. In these pellets the activity of lipase is similar to or slightly higher than the activity of amylase [61]. Besides pancreatin also lipase is added in the digestion model, which more than doubles the lipase activity (from 8.3 U/ml to 19.8 U/ml) in the chyme. This activity is 15 to 100-fold lower than reported in vivo [7, 9, 10, 13]. However, in other *in vitro* digestion models similar concentrations of pancreatin were used to mimick the fed state (in mg but corrected for USP activity): 3.9 mg/ml [32A en 32B], 1.32 mg/ml [31], 1 mg/ml [30], 1 mg/ml (fasting conditions) [33], 2 mg/ml [24]. Although this difference in activity with the in vivo situation, we propose, based on the 2.5 to 5-fold increase in vivo in response to food, to increase the amount of pancreatin to 3 mg/ml chyme and to increase the amount of lipase to 0.5 mg/ml chyme. The amount of lipase may be further increased to 1 mg/ml in case of a fat, high caloric meal. Furthermore, mucin may be added to the pancreatic juice to simulate the secretion of mucus by the Brunner glands in the small intestine (chapter 2).

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