Contaminants in food supplements and associated health risks

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CHAPTER 1
General introduction

Introduction

At present, there is an increasing interest in the use of food supplements including supplements made from natural sources such as for example clay or plant ingredients. Such use may result in intake levels that exceed the normal dietary intake of bioactive ingredients and/or contaminants present in the food supplements. Although food supplements are widely marketed, the safety of these preparations, especially when containing other ingredients than nutrient sources such as vitamins or minerals, is not generally assessed before they enter the market. Regulatory bodies have become more aware of this and are increasing their efforts to ensure the safety of food supplements. The 'Nederlandse Voedsel en Waren Autoriteit' (NVWA) (Netherlands Food and Consumer Product Safety Authority) performs the official controls on the safety of food supplements that are on the market in the Netherlands. Therefore, selected food supplements are analyzed by the NVWA or by other laboratories for contaminants.

The aim of the present PhD study was to investigate the presence and actual levels of selected contaminants in selected food supplements on the Dutch market and to estimate the associated health risks. The selection of food supplements for the studies in the present thesis was based on reports in literature such as the presence of metals in clay products for oral use (Abrahams 2002; Bakraji and Karajou 2003; Abrahams et al. 2006; Al-Rmalli et al. 2010; RASSF Number Notification 2002/336), the presence of polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene (BAP) in edible oils and food supplements (van der Wielen et al. 2006; Martena et al. 2011), the presence of dioxins in the feed chain resulting from the use of kaolinic clay for the sorting process of potatoes (Hoogenboom et al. 2010), and the presence of active pharmaceutical ingredients (APIs) in herbal food supplements such as the presence of APIs with aphrodisiac properties and APIs with weight reducing properties in herbal supplements (Blok-Tip et al. 2004; Zou et al. 2006; Wang et al. 2008; Chen et al. 2009). Therefore, the categories of foods and food supplements selected for the studies in the present thesis were:

1) clay products for oral use,
2) herbal food supplements claiming to enhance sexual potency,
3) herbal food supplements used for weight loss.

Furthermore, the food supplements selected for the present thesis are generally considered safe by the consumers using them, especially because they are of natural origin, but can contain contaminants at levels that can be cause for concern. The three categories of food supplements selected for the studies in the present thesis are regularly included in surveys by the NVWA because of their presumed use by vulnerable groups such as child bearing and lactating women, and people who are suffering from any disease linked to erectile dysfunction, or being overweight or suffering from obesity. The research presented includes quantification of selected contaminants of concern in

the respective food supplements but also an analysis of associated risks. To put the studies in perspective the subsequent sections in this introduction chapter describe the selected food supplements investigated as well as the contaminants analyzed and their legal framework, thereby providing the background for the work performed.

Ad 1) Clay products for oral use

The practice of eating clay or soil is termed geophagy. It is reported that clay and products of clay are used in traditional society by pregnant and lactating women and children mainly on the African continent, in Asia, and in North and South America (Halsted 1968; Vermeer 1971; Abrahams and Parsons 1996; Mahaney et al. 2000). These clay products, termed as traditional clay products, have African names such as calabash chalk, white clay, calabar stone, la craie, argile, nzu, mabele, emumba and ulo, Bengali names such as sikor, mithi, patri, khuri, kattha, poorcha or slatti, or a Suriname name which is pimba (Vermeer 1971; Hunter 1993; Mahaney et al. 2000; Reilly and Henry 2000; Smith et al. 2000; FSA 2002; Bakraji and Karajou 2003; Abrahams et al. 2006; De Korte 2006; Yanai et al. 2009; Al-Rmalli et al. 2010). Figures 1a and 1b show examples of clay products.

Use of clay products is influenced by social and cultural factors, such as culturally ingrained food preferences, supplementing the diets for spiritual reasons or to promote fertility, while some pregnant women use these products against morning sickness or to prevent miscarriage (Danford 1982; Abrahams and Parsons 1996; Aufreiter et al. 1997; Geissler et al. 1999; Henry and Matthews Kwong 2003; De Korte 2006; Kawai et al. 2009). Immigrants from the continents and regions mentioned above, also brought these habits to Western society such as to the United Kingdom (FSA 2002; Bakraji and Karajou 2003; Abrahams et al. 2006; Al-Rmalli et al. 2010; Katulek et al. 2010), the United States (Halsted 1968; Danford 1982; Katulek et al. 2010), Austria (Katulek et al. 2010), Belgium (Katulek et al. 2010) and the Netherlands (Schuttelaar & Partners 2003). In the Netherlands these clay products are generally sold in small ethnic shops and at markets (Schuttelaar & Partners 2003). Contamination of clay products for oral use is a recognized problem. Contaminants include toxic constituents such as the metals lead, mercury, and cadmium, the metalloid arsenic, persistent organic pollutants, microbes or parasites (Abrahams 2002; FSA 2002; Dean et al. 2004; Al-Rmalli 2010; Katulek et al. 2010).

a)

b)

Figure 1. Pictures of some clay preparations including **a)** sticks and **b)** pimba balls.

The occurrence of metals and the metalloid arsenic in clay products intended for oral use and the associated health risks.

Metals and metalloids occur naturally in various levels in the environment such as in water, soil and atmosphere, but can also be present in the environment as a result of human activities such as farming and industry. People are exposed to metals and metalloids by ingestion of food, water and soil. Accumulation of metals and metalloids in the body can lead to detrimental effects over time. For example, adverse health effects of exposure to lead and mercury could be brain damage. Young children are the most vulnerable group for these adverse health effects (EFSA 2004; 2010). Skin defects, development of skin cancer and induction of lung and urinary bladder cancer are reported as toxicological effects of exposure to inorganic arsenic (EFSA 2009) and kidney damage could be a toxicological effect of exposure to cadmium (EFSA 2012). Various authors reported levels of metals and metalloids such as lead, arsenic, cadmium and mercury in traditional clay products (Ibeanu et al. 1997; Dean et al. 2004; Al-Rmalli et al. 2010; Katulek et al. 2010). In 2002 there was an alert by EU-Member States to the presence on the Belgian and German market of calabash chalk with high lead contents (RASSF Number Notification 2002/336). In chapter 2 of the present thesis the occurrence and connected risk assessment of the selected metals and metalloid in clay products on the Dutch market are explored.

Contamination of African traditional pregnancy clay products with dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans).

The name 'dioxins' is often used for the family of structurally and chemically related polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Figure 2).

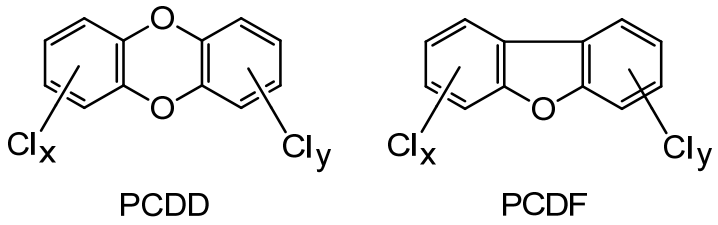

Figure 2. Chemical structures of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

Certain dioxin-like polychlorinated biphenyls (PCBs) with similar toxic properties are also included under the term 'dioxins' (WHO 2010[a]). The 17 dioxins which are of con-

cern are non-polar and persistent compounds that accumulate in the environment (sediment and soil) as well as in the fatty tissues of animals and humans (RIVM 2009; WHO 2010[a]). The aromatic rings of the structures of dioxins can be chlorinated to varying degrees. PCDDs and PCDFs can have up to 8 chlorine atoms substituting for hydrogen atoms (WHO 2010[b]). The WHO reported in 2010 that 419 dioxin-related compounds have been identified. Only about 30 of these 419 are considered to have significant toxicity, with 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) (figure 3) being the most toxic congener (WHO 2010[a]).

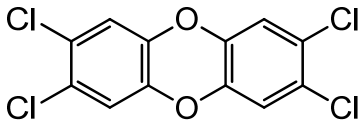

Figure 3. Chemical structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Human exposure either through food or the environment usually involves a complex mixture of dioxins and dioxin-like compounds, with each having its own degree of toxicity. In order to assess the potential toxicological risk of the whole mixture, the concept of toxic equivalence has been applied to this group of contaminants. TCDD, the most toxic member of the family, is used as reference compound, and all other dioxins are assigned a toxic potency relative to TCDD, based on experimental studies (WHO 2010[a]). The relevant dioxins have similar toxicity profiles and a common mechanism of action via interaction with the cellular Aryl hydrocarbon (Ah) receptor. Exposure of humans to high levels of dioxins may result in skin lesions, such as chloracne and patchy darkening of the skin, and altered liver function. Furthermore, exposure to dioxins is linked to impairment of the immune system, the developing nervous system, the endocrine system and reproductive functions (Van den Berg et al. 1998; White and Birnbaum 2009). Numerous animal studies with TCDD showed effects on the developing fetus, including a decreased sperm production of male rats exposed in utero (Faqi et al. 1998, Van den Berg et al. 2012), and these effects may require only a short-term exposure during a critical window of development. Dioxins are usually generated in a number of thermal and industrial processes as unwanted and often unavoidable by-products. In clay, the observed PCDDs are thought to have been formed under natural conditions in prehistoric ages, possibly by forest fires, volcanos or under high pressure and temperature in specific layers in the earth (Holmstrand et al. 2006; Gu et al. 2008; Horii et al. 2008).

The presence of PCDDs in clay was previously reported for clays applied in the food chain (Rappe et al. 1998; Hayward et al. 1999; Ferrario et al. 2000; Jobst and Aldag 2000). Our survey on dioxins in clay products was started in 2004, after an NVWA alert

of a French fries company in the Netherlands which changed its production process and used kaolinic clay for the sorting of potatoes. A by-product, small potato peels, contaminated with the clay, was fed to dairy cows at sone farms. Subsequently, high levels of PCDDs were detected in the fat of the milk obtained from these cows. It appeared that the absorption and carry-over of PCDDs from the clay to cow's milk was very efficient (Hoogenboom et al. 2010). Knowing that also human milk can be an important route for excretion of PCDDs (Malisch et al. 2010), the question was raised if dioxins would be present in traditional clay products that are used during pregnancy. We therefore analyzed Suriname and African traditional clay products, which were collected on the Dutch market, for the presence of dioxins. We also included some extra traditional clay products that were collected in several African countries. The occurrence and assessment of the potential risk due to dioxin exposure resulting from the use of contaminated clays during pregnancy are explored in chapter 3 of the present thesis.

Ad 2) Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supplements for the enhancement of sexual potency

Herbal food supplements, claiming to enhance sexual potency, may contain deliberately added active pharmacological ingredients (APIs) that can be used for the treatment of erectile dysfunction (ED), such as the APIs sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors. These APIs are used in synthetic drugs marketed for treating ED, such as for example in the drugs Viagra® containing sildenafil (Figure 4a) as API, Cialis®, containing the API tadalafil (Figure 4b) and Levitra®, containing the API vardenafil (Figure 4c).

Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors have their mode of action on the nitric oxide–cyclic guanosine monophosphate (cGMP)-specific PDE-5 enzyme (Singh et al. 2009; Venhuis and de Kaste 2012). The PDE-5 enzyme is present in the lining of the blood vessels supplying the corpus cavernosum penis, which is the erectile tissue of the penis. As a result of this, use of PDE-5 inhibitors increase the blood flow to the penis during sexual stimulation (Corbin et al. 2002; Pissarnitski 2006). Therefore, in medical practice PDE-5 inhibitors are used to treat erectile dysfunction. ED is defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance (NIH 1993).

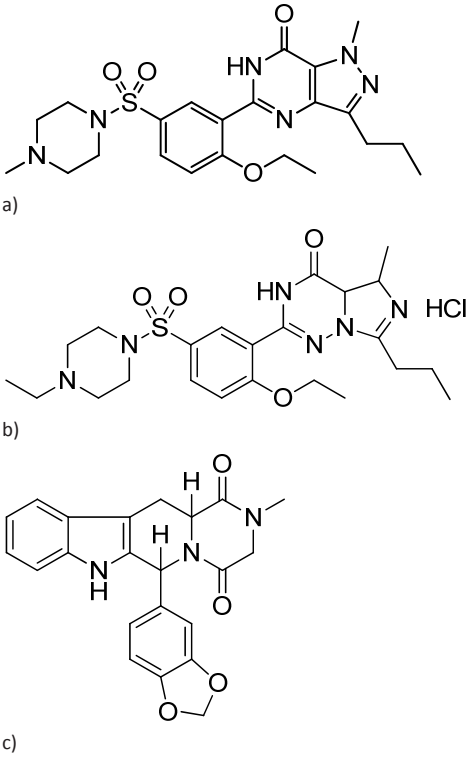

Figure 4. Chemical structures of **a)** sildenafil **b)** tadalafil and **c)** vardenafil.

The PDE-5 enzyme is also present in the arterial wall of the smooth muscles within the lungs. PDE-5 inhibitors give a selective vasodilatation of the pulmonary blood vessels and are therefore used in medical practices to decrease pulmonary hypertension (PH). Revatio® (Pfizer) and Adcirca® (Lilly) are examples for drugs marketed for PH, and include the PDE-5 inhibitors sildenafil and tadalafil, respectively. Use of PDE-5 inhibitors is contra-indicated in patients who are treated with other types of antihypertensives such as nitrates (eg. nitroglycerine, doxazosin and terazosin) (Boden et al. 2012; EMA 2013). PDE-5 inhibitors are also contra-indicated in patients suffering from hypotension, but who are not treated for this (Kloner 2007). After the NVWA was alerted to the presence on the Dutch market of herbal food supplements intended for ED and containing sildenafil, the NVWA started a continuous survey in 2002. Subsequently, illegal APIs such as analogous PDE-5 inhibitors were identified in herbal food supplements marketed for ED. Most of these APIs are not registered drug substances and have not been tested for their safety. Often these APIs are by-products of the discovery process for the original drug carried out by the pharmaceutical industry. To illustrate the scale of this illegal practice in food supplements on the market one should consider that litera-

ture shows that until 2011 at least 46 different analogues of sildenafil, vardenafil and tadalafil were detected in food supplements (Venhuis and de Kaste 2012). The food supplements to which this relates are reported to be present on the market in countries such as India, Taiwan, Singapore, the United States of America and the Netherlands and are often obtained via Internet shops (Lin et al. 2006; Zou et al. 2006; Singh et al. 2009; Venhuis and de Kaste 2012; FDA 2013). The occurrence and connected risk assessment of sildenafil and analogues of PDE-5 inhibitors in herbal food supplements is explored in chapter 4 of the present thesis.

Ad 3) Sibutramine, desmethylsibutramine, didesmethylsibutramine, rimonabant and phenolphthalein in herbal food supplements used for weight loss

Nowadays, overweight and obesity are considered a growing public health issue with more than 1.4 billion adults being overweight. Overweight is a status that can be defined by a body mass index (BMI) between 25–30 kg m^{-2}. The WHO estimates that at least 500 million people are obese (BMI greater or equal to 30 kg m^{-2}) (WHO 2013). In the past a limited number of approved pharmaceuticals to treat obesity and overweight were available on the market.

The general public uses many other methods for weight loss including herbs, vitamins, nutritional supplements, and meal replacement preparations (Hassini-Ranjbar et al. 2009) and this includes the wide variety of herbal food supplements intended for weight loss present on the market. In the Netherlands, the presence of sibutramine and phenolphthalein in a vitamin supplement and in capsules was first reported by the National Customs Laboratory in 2004 (RIVM 2009). Based on frequent reports of adulterations and side effects resulting from the use of herbal food supplements for the treatment of overweight, obesity and constipation, shown to contain APIs such as sibutramine, fenfluramine, rimonabant, orlistat, and phenolphthalein (Yuen et al. 2007; Zou et al. 2007; Wang et al. 2008; Chen et al. 2009; RIVM 2009; Tang et al. 2010; Vaysse et al. 2010; Stypulkowska et al. 2011; De Carvalho et al. 2012; Dunn et al. 2012; Phattanawasin et al. 2012; Ancuceanu et al. 2013), and reports by health authorities such as from the Netherlands (IGZ 2010), Australia (Australian government 2007), Canada (Health Canada 2006), and the UK (MHRA 2010), the NVWA started also a survey on herbal products intended for weight loss and identified the presence of APIs such as sibutramine, desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant and the laxative phenolphthalein.

Sibutramine, the chemical structure of which is shown in figure 5a, is a combined serotonin (5-HT) and noradrenaline (NA) re-uptake inhibitor. This API was prescribed until 2010 in regular medical practice to manage obesity. The increased levels of neurotransmitters, serotonin and noradrenalin in the brain helped patients to feel full after a

meal, and this helps to reduce their food intake. Sibutramine was suspended from marketing in the EU in 2010 because of risk of serious, non-fatal cardiovascular events, such as stroke or heart attack and also because of the rebound of weight gain after termination of therapy (EMA 2014).

In chapter 5 of the present thesis we investigated the presence of DMS and DDMS, of which sibutramine is a prodrug, in a series of selected herbal supplements. The chemical structures of DMS and DDMS are shown in figures 5b and 5c. We also quantified the API rimonabant (Figure 5d), which is a selective cannabinoid-1 receptor (CB1) blocker (Van Gaal et al. 2005; Padwal and Majumdar 2007). Furthermore, the laxative phenolphthalein, which is a benzofuran derivate and of which the chemical structure is shown in figure 5e, was quantified in these herbal supplements. In 1996, the US National Toxicology Program (NTP) published data on the genotoxity of phenolphthalein and its carcinogenicity in laboratory animal studies (NTP 1996). The EMA concluded that the National Competent Authorities should take these NTP data into account in their considerations of any restriction of phenolphthalein containing medicinal products on the national markets (EMA 1997).

The presence of these APIs in herbal supplements characterized the third group of food supplements investigated in the present thesis. The occurrence and connected risk assessment of the presence of sibutramine, DMS, DDMS, rimonabant, and phenolphthalein to herbal food supplements for weight loss is explored in chapter 5 of the present thesis.

It should be noted that the views expressed in this thesis are those of the author and co-authors and do not reflect the official policy or position of the NVWA, and/or any scientific institute and/or any Ministry of the Netherlands.

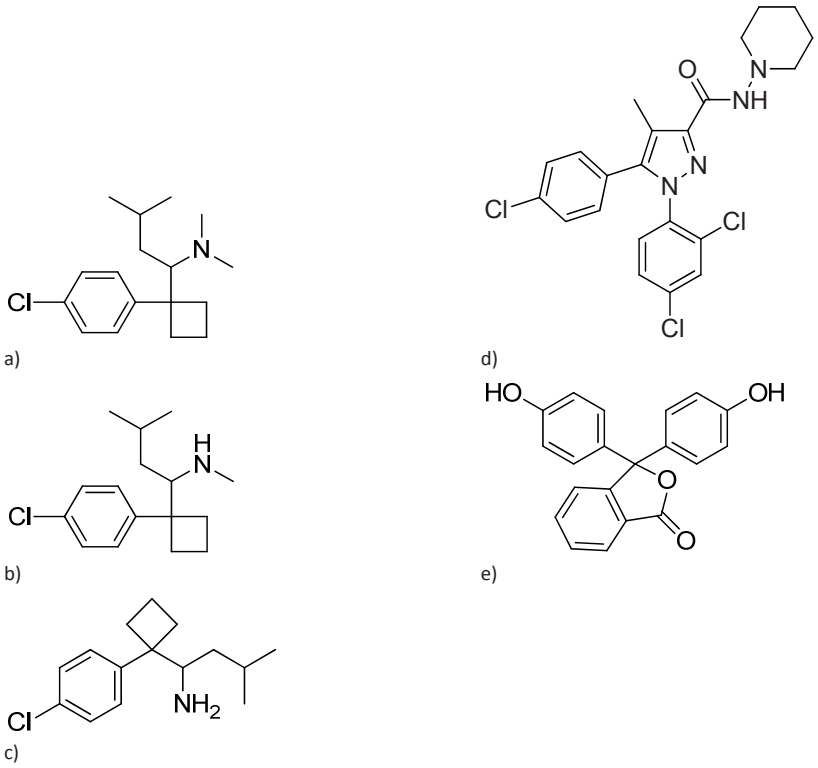

Figure 5. Chemical structures of **a)** sibutramine **b)** desmethylsibutramine **c)** didesmethylsibutramine **d)** rimonabant, and **e)** phenolphthalein.

Legal framework

In the next sections a short overview is presented of the legal framework that covers the products of concern.

Ad 1) Clay products for oral use

The European Food Law and the Dutch Commodities Act, of which the latter is based to a large extent on European Food Law, have specific legislation regarding food supplements such as vitamins, minerals, and herbal preparations. Dietary supplements may be presented in a pharmaceutical form (e.g. tablets, capsules), though dietary supplements may not claim to be medicines. Furthermore, dietary supplements require no pre-marketing evaluation for safety or efficacy (VWS 2003). Of the clay products includ-

ed in the studies presented, only health clay products are covered by this definition of food supplements because these products are marketed in dose form. Traditional clay products are mostly not sold in dose form but rather without labelling as powder, sticks or balls (see figures 1a and 1b). Therefore, traditional clay products can formally not be considered to be food supplements. For the different metals and the metalloid arsenic, enforcement on both categories of clay products was initially based on the legal requirement laid down in article 14.1 of Regulation (EC) No. 178/2002 indicating that food shall not be placed on the market if it is unsafe (EC 2002). In the course of our study, the European Commission established maximum levels for lead, mercury, and cadmium in food supplements in Regulation (EC) No. 1881/2006 (EC 2008). These EC maximum levels could be applied to the health clay products, given that only these clay products can be considered to be food supplements.

Ad 2 and 3) Herbal food supplements

The Dutch Commodities Act Decree 'Herbal preparations' (in Dutch 'Warenwetbesluit Kruidenpreparaten) covers herbal preparations that are brought on the market as foods and non food products (VWS 2001). The decree defines herbal preparations as 'herbal substances, subjected to treatment or not, including herbal extracts, which are intended to be used by humans'. Herbal substances and herbal preparations from which it is plausible that these herbal substances or preparations are designated to be processed or further processed to medicines are not covered by this decree because medicines are outside the scope of this decree. In the General Food Law medicinal products are excluded from the definition of food (EC 2002). Herbal supplements are not subjected to strict regulation for medicines (Venhuis et al. 2008), and recent examples such as the presence of lipid-lowering and weight reducing agents, and APIs with sedative, antidiabetic, hormonal and aphrodisiac properties in herbal supplements (Chen et al. 2009; Toriaans et al. 2010) show that herbal supplements, which are covered by the definition of food, are apparently an ideal matrix for adding APIs. A product can fall within the definition of a medicinal product when it is capable of appreciably restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action (European Court of Justice 2009). A product can also fall within this definition when it is presented as having properties for treating or preventing disease in human beings by for instance using a medical claim in an advertisement for a product. Enforcement of herbal food supplements containing APIs could take place by applying the Dutch Commodities Act or the Dutch Medicines Act. Enforcement by the Dutch Commodities Act is based on the legal requirement that a food shall not be placed on the market if it is unsafe, as laid down in article 14.1 of Regulation (EC) No. 178/2002. Therefore, when applied to herbal supplements with APIs a risk manage-

ment authority needs to prove that the herbal supplement is unsafe, based on the intake levels of the API when the product is used as intended. One could also explore an enforcement strategy for herbal supplements using the Dutch Medicines Act because herbal supplements containing APIs could fall within the definition of a medicinal product based on its pharmaceutical activity. Medicinal products are subject to approval before marketing, and herbal products that fall within the definition of medicinal product have often not been approved. In order to investigate whether a product is a medicinal product based on its pharmaceutical activity it is essential to prove that the observed levels can result in pharmacological effects. For both enforcement scenarios, i.e. the one based on the Dutch Commodities Act and the one based on the Dutch Medicines Act, the safety or pharmacological effects of the herbal supplement containing APIs when used as intended should be assessed.

Objective and outline of this thesis

The aim of the present PhD project was to investigate the presence and actual levels of various contaminants and pharmacologically active substances in selected food supplements on the Dutch market and to estimate the associated health risks. This was done for clay products for oral use, herbal food supplements for the enhancement of sexual potency and herbal food supplements used for weight loss.

Chapter 1 of this thesis, the present chapter, provides a general introduction to safety concerns about clay products for oral use, herbal preparations for the enhancement of sexual potency and herbal weight loss food supplements that were brought on the market as food commodities in the Netherlands. It also provides a short introduction on legislation and regulation relating to these products.

Chapter 2 discusses the results of investigations into the occurrence of selected metals and the metalloid arsenic in clay products intended for oral use and the associated risks.

Chapter 3 focuses on the contamination of traditional clay products, which are generally used during pregnancy, with polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-furans and the associated risks.

Chapter 4 presents data on sildenafil and analogous PDE-5 inhibitors in herbal preparations used for the enhancement of sexual potency, and the evaluation of associated risks.

Chapter 5 investigates the contamination with sibutramine, desmethylsibutramine, didesmethylsibutramine, rimonabant and phenolphthalein in herbal preparations used for weight loss, and evaluates associated risks.

Chapter 6 presents a summary of the results obtained in this thesis and provides a discussion on how these results can be translated to the safety of food supplements currently on the Dutch market.

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CHAPTER 2

Levels of lead, arsenic, mercury and cadmium in clays for oral use on the Dutch market and estimation of associated risks

Based on:
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Levels of lead, arsenic, mercury and cadmium in clays for oral use on the Dutch market and estimation of associated risks

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Abstract

Pregnant women in Africa, Asia and Suriname, and some immigrants in western societies traditionally consume clay products known by a variety of names such as mabele, calabash chalk, sikor and pimba. Furthermore, clay is used for health purposes in western societies. Because certain clays can contain high levels of metals and metalloids, the aim of this study was to determine lead, arsenic, mercury and cadmium in clay products for oral use available on the Dutch market. Traditional clays originating from Africa (n=10) and Suriname (n=26) and health clays (n=27) were sampled from 2004 up to and including 2012.

Total metal and metalloid contents were measured by ICP-MS and showed maximum levels of lead, arsenic, mercury, and cadmium of 99.7, 45.1, 2.2 and 0.75 mg kg^{-1} respectively. In the absence of maximum limits for these type of clays, the potential exposure was estimated from the determined concentration, the estimated daily use level of the clays and the estimated bioaccessibility of the different metals and arsenic. The intake estimates were compared to existing health based guidance values. For lead, the use of 34 of the 36 traditional clays and two of the 27 health clays would result in intake levels exceeding the toxicological limit by up to 20-fold. Use of 15 of the 35 traditional clays and 11 of the 27 health clays would result in intake levels exceeding the toxicological limit for inorganic arsenic by up to 19-fold. Although limited bioaccessibility from the clay may limit the exposure and exceedance of the health based guidance values, it was concluded that lead and arsenic intakes from some clay products could be of concern also because of their use by pregnant women and the potential developmental toxicity. As a result the use of these products, especially by pregnant women, should be discouraged.

Introduction

Geophagy, the practice of eating clay or soil, is observed among people of many continents. This practice has mainly been reported for children and for pregnant and lactating women in parts of the African continent, in Asia, and in South and Central America (Halsted 1968; Vermeer 1971; Abrahams and Parsons 1996; Mahaney et al. 2000). The eating of clay or soil is determined by social and cultural factors. However, the medical profession considers geophagy as an aberrant behaviour, and potentially hazardous because of the presence of toxic constituents in the material such as metals and metalloids, persistent organic pollutants, microbes and parasites (Abrahams 2002; Katulek et al. 2010). Clay can be defined as the fine fraction of a soil and contains particles of less than 2 µm size (Soil Survey Division Staff 1993). In Africa the habit of consuming clay is practiced by pregnant women against morning sickness, for supplementing the diet, and for spiritual reasons, but also to promote fertility in non-pregnant women (Danford 1982; Abrahams and Parsons 1996; Aufreiter et al. 1997; Geissler et al. 1999; Henry and Matthews Kwong 2003; Kawai et al. 2009). Examples of the large variety of names used for these clay products at local markets in African countries like Ghana, Uganda, Tanzania, and Zambia are calabash chalk, white clay, calabar stone, la craie, argile, nzu, mabele, emumba and ulo (Vermeer 1971; Hunter 1993; Reilly and Henry 2000; Smith et al. 2000; FSA 2002; Yanai et al. 2009). Women in Java and in the Bengal area of South Asia are also reported to consume clay products during pregnancy. The products from the Bengal area are available in the form of powder or a solid tablet form with names like sikor, mithi, patri, khuri, kattha, poorcha or slatti (Mahaney et al. 2000; Bakraji and Karajou 2003; Abrahams et al. 2006; Al-Rmalli et al. 2010). In Suriname, pimba, balls of white or reddish clay, are eaten by some pregnant women of the Creole society. Pimba is used orally to prevent miscarriage, treat acid reflux or promote delivery and it is also applied to the skin for spiritual reasons (De Korte 2006).

Immigrants from Africa, Java, Bengal area, and Suriname have brought the traditional practice of clay consumption to western countries. In the United Kingdom for instance, different sorts of clay are consumed by the Nigerian and the wider West-African Community and in Asian communities (FSA 2002; Abrahams et al. 2006; Al-Rmalli et al. 2010). Immigrants from Mexico have brought this practice into the United States as well (Halsted 1968; Danford 1982). In the Netherlands, pregnant women from Suriname are reported to consume pimba (Schuttelaar & Partners 2003). Clay products originating from Africa, Asia and Suriname are generally sold in small ethnic shops and at markets in the Netherlands. Often labelling of these products is poor, or in many cases absent, and the intended use is not specified.

In addition to its traditional use, clay is also marketed in capsules, tablets or powdered dose form as health food. These health clay products are generally well packaged and labelled and are widely available in health- and Internet shops in the Netherlands. Health clays are claimed to protect the gastrointestinal tract and aid detoxification and are also recommended for use during pregnancy.

In literature increased intakes of lead and arsenic due to geophagy were reported (Abrahams 2002; Bakraji and Karajou 2003; Abrahams et al. 2006; Al-Rmalli et al. 2010). Especially lead readily crosses the placenta, and poses a risk to the unborn child, because it may interfere with the neurological development of the fetus (Baars et al. 2001). In 2002, EU-member states were alerted through the EU-Rapid Alert System for Food and Feed to the presence on the Belgian and German market of calabash chalk with high lead contents ranging from 8.2 up to 16.1 mg kg^{-1} (RASSF Number Notification 2002/336). Subsequently, the Netherlands Food and Consumer Product Safety Authority (NVWA) identified similar traditional clay products intended for oral use on the Dutch market. At a later stage, clay products marketed as health foods were analyzed for lead, other metals and the metalloid arsenic. This paper presents the results of a survey on lead, arsenic, mercury and cadmium, and in addition some other components like aluminum, magnesium, copper, chromium, iron and zinc in clay products for oral use collected on the Dutch market from 2004 up to and including 2012. Exposure levels to these metals and the metalloid arsenic were estimated using the analytical data and the estimated or recommended daily dose levels of the individual clay products. Since absorption of metals from soil including clay may be rather poor in comparison to the salts used in toxicological studies, the risk assessment was refined by applying estimates of the bioaccessibility of the different metals and the metalloid arsenic for soil.

a)

b)

Figure 1. Pictures of some clay preparations including **a)** sticks and **b)** pimba balls.

Material and methods

Sampling

For this study NVWA inspectors sampled 63 clay products from 2004 up to and including 2012. Sampling locations were selected from the NVWA inspection database or identified by an Internet search. All traditional clay products (n=36) were collected from importers and vendors of ethnic foods throughout the Netherlands, and were exclusively of African and Surinam origin. The clay products which we included in our study are likely to be mixtures of different soil types with different particle sizes and consisted of pellets, balls, sticks, and powder. Only products for oral use were included.

Since traditional products were not labelled, vendors were asked to confirm that the products were intended to be used orally. Figures 1a and 1b show two types of traditional clay products. These products were only available without label. The vendor identified the product shown in Figure 1b as pimba for use against morning sickness in the first trimester of pregnancy. Health clays were sampled in health shops. All samples of health clays (n=27) were pre-packaged and in the form of powder, capsules, tablets or a liquid solution. Except for three health clay products, the label included instructions for use. As stated on the label several health clay products were intended to be used by pregnant women.

ICP-MS determination of metals and the metalloid arsenic

A routine method for the determination of metals and metalloids in clays for oral use based on Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was developed and validated for enforcement purposes for lead, arsenic, mercury and cadmium. In addition, aluminum, magnesium, copper, chromium, iron, and zinc were also analyzed. However, the analytical method employed for this was not validated. Samples were completely homogenized by grinding them to powder with a grinder (Retsch, Haan GmbH, Germany). To 200 mg of the powdered sample 3 ml nitric acid (HNO_3, 65%) and 3 ml hydrochloric acid (HCl, 30%) were added. Digestion of the samples was then performed using a microwave oven (Ethos Plus Microwave Labstation, Milestone, Italy) in which the samples were treated for 25 minutes at 200^0C. The solution produced by the digestion process was then quantitatively transferred with de-ionized water from the digestion vessel to a 100 ml volumetric flask. Because of the presence of particles, the solution was filtered while transferring it to the volumetric flask. Throughout the analysis Milli-Q grade de-ionized water was used. The sample solutions were measured with ICP-MS (Thermo Electron PQ Excell, Thermo Scientific, United Kingdom) using the 'Collision Cell Technology mode' (CCT-mode) with a helium/hydrogen flow (95/5%).

Using this gas mixture in the CCT-mode most interferences were reduced (Van der Wielen et al. 2005). In order to test if each vessel was clean prior to use, test runs were performed on each vessel with only 3 ml HNO_3 (65%) and 3 ml HCl (30%) before each series of samples. This was repeated after each series in order to confirm that no detectable metalloid or metals were left in the vessels. For the in house validation of the method, first the limits of detection and quantification (LOD and LOQ, respectively) were determined and it was checked if these fulfilled the requirements of Commission Regulation (EC) No. 333/2007. The LOQ was defined as half the concentration of the lowest calibration standard. The LOD was defined as half the LOQ. The range of the calibration curve was used to calculate the range of application. When necessary, samples were diluted and reanalyzed to fit within the range of the calibration curve. The accuracy of the method was determined by analysis of a soil Certified Reference Material (CRM) (LGC Standards; CRM24-050 – Metals on Soil), which contained certified levels of the metalloid and metals analyzed. The results of this CRM analysis are required to be within the 95% confidence interval of the corresponding certified values. Furthermore, the relative standard deviation (RSD) was measured by replicate analysis (n=6) of the CRM under repeatability conditions. The CRM was also used as quality control sample in each series of measurements. The expanded measurement uncertainty (in mg kg^{-1}) was determined as the reproducibility from measurements (n>10) of this CRM, analyzed by different persons on different days. Table S-1 lists the following method performance characteristics LOD, LOQ, range of application, recovery and the RSD for repeatability. The recovery was only determined for lead, arsenic, mercury and cadmium. According to the 'AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals', the recovery has to be between 75 and 120% at the measured concentrations (AOAC 2002). The values for the recovery of the method for cadmium, mercury, arsenic and lead that are listed in Table S-1, fitted well within these limits. For the other metals that can be analyzed with this method the recovery has not been determined. The RSD values for lead, arsenic, mercury, cadmium, aluminum, magnesium, copper, chromium, iron and zinc, with arsenic displaying the highest value of 13.0%, were found to be below the required 15% at the measured concentrations (AOAC 2002). To overcome the build up of mercury residues in the system, AuCl was added to the sample and rinse solutions. AuCl was selected for this purpose because of the capability of mercury to complex with gold (Falciani et al. 2000).

Table S-1. Method performance characteristics of the ICP-MS analysis for different metals and metalloid, determined by in house validation.

Metal/metalloid	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Range of application (mg kg^{-1})	Range of application (mg $week^{-1}$)	Recovery (%)	RSD[a] (%)
Aluminum	0.63	1.25		0.14 – 3.7	Not validated	7.0
Arsenic	0.063	0.125		0.01 – 0.37	89.7	13.0
Cadmium	0.063	0.125	0.03 – 1.25	0.009 – 0.37	95.9	5.8
Chromium	0.063	0.125		0.015 – 0.37	Not validated	9.5
Copper	0.63	1.25		0.1 – 3.7	Not validated	6.6
Iron	0.63	1.25		0.1 – 3.7	Not validated	7.0
Lead	0.05	0.10	0.10 – 1.25	0.03 – 0.37	93.9	3.3
Magnesium	0.63	1.25		0.1 – 3.7	Not validated	6.6
Mercury	0.013	0.025	0.006 – 0.25	0.002 – 0.074	97.3	5.8
Zinc	0.63	1.25		0.16 – 3.7	Not validated	6.7

Note:

[a] Relative Standard Deviation for repeatability

Estimation of the weekly intake of metals and the metalloid arsenic, selection of health based guidance values, and estimation of bioaccessible fractions

For traditional clay products, we used 42 g as the estimated daily dose level, which was defined using reports on geophagy in different countries as an estimate for the daily dose level of traditional clay products (Reeuwijk et al. 2013). This estimate is based on a study by Geissler et al. (1999) on dose levels used in Kenya in which a median daily dose level of 41.5 g day^{-1} was determined. To calculate the estimated daily intake of the selected metalloid and metals with a health clay product we applied the highest recommended dose level per day stated on the label of the product, which ranged from 1.5 to 35.1 g day^{-1} (Table S-2). For three health clay products no daily dose levels were recommended on the label and therefore for these products estimated daily dose levels of 7 g day^{-1} were used, which is the mean of the recommended dose levels identified on labels of the other health clay samples. In the Netherlands, consumers of African origin, who use traditional clays, estimated the daily consumption of calabash chalk by pregnant women in Africa to be 20 clay sticks. However, daily use levels in the Netherlands were estimated to be ten times less (Schuttelaar & Partners 2003). Although daily use levels in the Netherlands might be lower than in Africa or other countries of origin, we maintained the default daily dose level of 42 g in order to protect those pregnant women in the Netherlands who adhere to use levels which have been reported for Africa or other countries of origin. RIVM/SIR (2003) and the Food Standards Agency (FSA) (2002) both estimated a daily dose level of calabash chalk of 60 g. No data on the dose levels of pimba from Suriname were identified in literature.

Table S-2. Contents of metals and arsenic in clay products and estimated or recommended daily dose level for individual clay products.

Origin of clay product[a]	Name clay product	Content (mg kg^{-1})										Daily dose (g day^{-1})
		Aluminum	Arsenic	Cadmium	Chromium	Copper	Iron	Lead	Magnesium	Mercury	Zinc	
Africa	Mabele	150000	10.6	0.452	128		14000	46.8	931			42
	Mabele sale	150000	2.75	0.430	67.3		6800	36.8	2460			42
	Mabele sale	78000	2.04	0.201	47.6	13	11000	37.4	3480	0.548	41	42
	Mabele non-sale	< LOQ	4.08	0.272	95.2	22	210000	19.2	18600	0.156	130	42
	Mabele Cameroon	120000	8.74	0.218	120	13	24000	47.6	1070	0.160	51	42
	Mabele	95000	2.13	0.201	71.4	15	14000	45.9	4210	0.561	54	42
	Mabele, Nigeria	88000	17.3	< LOQ	110	41	37000	27.5	7480	0.299	37	42
	Mabele whole, Zaïre	< LOQ	10.9	0.418	81.6	61	32000	38.1	13900	0.0646	220	42
	Mabele broken, Zaïre	< LOQ	8.50	0.173	110	18	12000	23.4	1830	2.20	48	42
	Mabele	87000	11.5	< LOQ	103	38	26000	19.3	6470	0.0630	35	42
Suriname	Pimba	180000	0.875	0.382	193		5100	23.9	211			42
	Pimba	210000	0.633	0.205	165		6200	56.5	183			42
	Pimba	190000	0.658	0.164	158		6000	59.3	156			42
	Pimba	180000	0.414	0.176	42.6		5000	28.1	65.9			42
	Pimba	150000	0.538	0.238	56.1		5600	59.4	67.6			42
	Pimba	190000	0.520	0.210	46.3		5400	32.6	81.6			42
	Pimba	200000	0.436	0.175	55.0		6200	99.7	75.7			42
	Pimba balls	200000	0.511	0.267	58.1		5400	30.0	90.5			42
	Pimba balls	190000	0.531	0.403	98.0		4300	28.6	132			42
	Pimba balls	180000	0.890	0.383	188		5000	35.2	200			42
	Pimba balls	180000	0.317		131		6000	21.7	395			42
	Pimba balls	180000	0.580	0.405	98.8		3900	23.1	144			42
	Pimba Doti							16.0				42
	Pimba	280000	27.2	0.201	4.97	41	7500	33.3	1660	1.82	29	42
	Pimba	140000	0.490	< LOQ	48.8	5.0	10000	30.2	83.2	0.0790	17	42
	Pimba	190000	1.02	0.142	43.2	5.9	6800	24.9	59	0.0530	17	42
	Pimba	170000	1.89	< LOQ	50.5	18	9100	35.2	4450	0.481	58	42
	Pimba	< LOQ	1.02	0.190	91.8	11	5100	50.0	106	0.221	34	42
	Pimba balls	120000	1.05	0.184	57.8	8.2	6500	30.3	106	0.194	15	42
	Pimba	130000	0.583	< LOQ	36.6	6.2	3900	25.6	47.4	0.0380	31	42
	Pimba	160000	0.523	< LOQ	50.0	5.3	6500	26.2	63.0	0.0560	18	42
	Pimba	170000	0.352	0.133	57.7	6.3	5500	25.8	76.7	0.0470	13	42
	Pimba	4400	3.85	< LOQ	6.6	4.3	2700	< LOQ	3500	< LOQ	5.9	42
	Pimba	160000	0.556	< LOQ	60.4	2.6	5700	30.0	154	0.0490	26	42
	Pimba	160000	12.5	< LOQ	121	33	42000	26.5	7220	0.0480	44	42
	Pimba	42000	5.38	0.296	9.22	5.1	11000	1.77	9020	0.248	10	42
Europe	Health Clay I[b]	1200	0.480	< LOQ	2.21	< LOQ		0.510		< LOQ	2.7	7
	Health Clay II[b]	1400	0.500	< LOQ	2.39	< LOQ		0.540		< LOQ	2.7	7
	Health Clay III[b]	78000	9.70	< LOQ	73.5		36000	15.0	13900			7

Origin of clay product[a]	Name clay product	Content (mg kg^{-1})										Daily dose (g day^{-1})
		Aluminum	Arsenic	Cadmium	Chromium	Copper	Iron	Lead	Magnesium	Mercury	Zinc	
	Health Clay IV[b]	6900	3.30	< LOQ	57.7	49	33000	22.3	6560	< LOQ	130	2.6
	Health Clay V[b]	19000	20.6	0.206	50.8	22	23000	14.8	11200	0.151	74	10.4
	Health Clay VI[b]	18000	19.0	0.201	45.8	21	25000	14.1	10600	0.123	70	12.8
	Health Clay VII[b]	24000	7.36	< LOQ	38.1	13	19000	9.78	6840	< LOQ	42	3.3
	Health Clay VIII[b]	< LOQ	13.1	0.750	59.5	26	29000	20.6	13300	0.476	92	12.0
	Health Clay IX[b]	< LOQ	10.4	0.357	66.7	20	39000	21.9	7680	0.107	79	12.0
	Health Clay X[b]	< LOQ	5.24	< LOQ	76.2	67	28000	8.57	7190	0.762	140	3.0
	Health Clay XI[b]	70000	10.5	< LOQ	55.3	20	38000	15.1	16000	< LOQ	84	2.2
	Health Clay XII[b]	32000	7.37	0.141	41.6	23	20000	11.7	10700	0.0490	72	12.6
	Health Clay XIII[b]	39000	9.52	0.139	31.7	24	19000	11.8	10900	0.0450	74	13.6
	Health Clay XIV[b]	57000	11.2	< LOQ	62.6	21	32000	15.4	23900	< LOQ	90	1.5
	Health Clay XV[b]	40000	5.71	0.214	76.1	39	33000	4.90	6740	< LOQ	100	6
	Health Clay XVI[b]	75000	29.0	0.639	205	40	51000	20.9	16000	0.109	110	4.4
	Health Clay XVII[b]	71000	11.7	< LOQ	57.9	16	38000	16.6	25300	< LOQ	90	4.4
	Health Clay XVIII[b]	200000	< LOQ	< LOQ	55.3	6.8	4600	31.4	57.0	< LOQ	18	3.2
	Health Clay XIX[b]	32000	7.02	0.145	25.6	20	20000	11.9	9950	0.0300	67	16.0
	Health Clay XX[b]	35000	8.78	0.136	53.2	21	27000	11.7	11700	0.0410	71	13.2
	Health Clay XXI[b]	22000	1.30	< LOQ	0.777	2.3	2100	3.07	2780	< LOQ	11	3.0
	Health Clay XXII[b]	75000	4.04	< LOQ	7.62	16	28000	19.9	63500	< LOQ	83	1.6
	Health Clay XXIII[b]	80000	45.1	0.352	81.5	36	46000	36.4	21900	0.0370	110	2.2
	Health Clay XXIV[b]	46000	5.62	0.180	60.8	48	48000	4.74	9820	0.0280	130	3.2
	Health Clay XXV[b]	30000	6.45	0.139	44.2	10	19000	11.0	11400	< LOQ	46	35.1
	Health Clay XXVI[b]	36000	5.81	< LOQ	70.2	32	37000	4.38	7680	< LOQ	85	2.8
	Health Clay XXVII[b]	40000	5.78	< LOQ	73.5	32	39000	3.81	7180	< LOQ	81	2.8

Notes:
LOQ = Limit of Quantification
[a] origin based on information gathered by NVWA inspectors
[b] brand names are blinded

For each metalloid or metal analyzed we selected an established health based guidance value by reviewing risk assessments by national and international bodies (Table S-3). Pertinent differences in toxicity between various chemical species of a metal or metalloid can exist (WHO 2006). When possible this was taken into account when selecting appropriate health based guidance values. Given that clay products are often consumed by pregnant women risk assessment was primarily aimed at adverse effects on the fetus. However, when health based guidance values for developmental effects were lacking, the risk assessment was based on other health based guidance values for other toxic effects relevant to the mother. These values could either be tolerable intake levels for adverse effects with a threshold or benchmark dose (BMD) values for adverse effects without a threshold. During our study a $BMDL_{01}$ value was set for lead (EFSA

2010). The $BMDL_{01}$ value represents the 95th percentile lower confidence limit of the BMD resulting in 1% extra risk (EFSA 2010). Although this BMDL value contains no extra safety values, it can be used to judge the safety of estimated exposure levels based on the margin of safety. The European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends using a margin of exposure (MOE) approach as priority setting for risk managers (EFSA 2005; JECFA 2005). The MOE is the ratio between the BMDL for the adverse effect and the estimated intake of the metal or metalloid and can be used. We applied the MOE approach for lead and arsenic.

Table S-3. Toxicological health based guidance values for different metals and metalloids.

Metal/ metalloid	Health based guidance value	Type of Health based guidance value	Health based guidance value set by	Health based guidance value set for	Health based guidance value (mg $week^{-1}$) [a]
Aluminum	1 mg kg bw^{-1} $week^{-1}$	PTWI[b];TWI[c]	JECFA (2007) ; EFSA (2008)	all aluminum containing additives in food	60
	2 mg kg bw^{-1} $week^{-1}$	**PTWI[b]**	**JECFA (2011[a])**	**All aluminum compounds in food. including food additives**	**120**
Arsenic	**1.0 µg kg bw^{-1} day^{-1}**	**TDI[d]**	**RIVM (Baars et al. 2001)**	**Inorganic arsenic**	**0.42**
	0.3- 8 µg kg bw^{-1} day^{-1}	**$BMDL_{01}$[e]**	**EFSA (2009[a])**	**$BMDL_{01}$ values for use in the risk characterization for inorganic arsenic for relevant health endpoints, i.e. 1 % increased risk for cancer in the lungs, skin and bladder and skin lesions.**	**0.13** - 3.36
	3.0 (range 2.0 - 7.0) µg kg bw^{-1} day^{-1}	$BMDL_{0.5}$[f]	JECFA (2011[b])	Inorganic arsenic $BMDL_{0.5}$ value for 0.5% increased risk on lung cancer	1.26 (range 0.84 – 2.94)
Cadmium	**2.5 µg kg bw^{-1} $week^{-1}$**	**TWI[c]**	**EFSA (2009[b])**	**Cadmium in food**	**0.15**
Chromium	**5 mg kg bw^{-1} day^{-1}**	**TDI[d]**	**RIVM (Baars et al. 2001)**	**Insoluble chromium (III) compounds and metallic chromium**	**2100**
	5 µg kg bw^{-1} day^{-1}	TDI[d]	RIVM (Baars et al. 2001)	Soluble chromium (III) compounds	2.1
	250 µg day^{-1}	UL[g]; Maximum Intake Level	WHO (1996); EFSA (2010[a])	Trivalent chromium as a nutrient added for nutritional purposes to foodstuffs for particular nutritional uses and foods intended for the general population (including food supplements)	1.75
Copper	**140 µg kg bw^{-1} day^{-1}**	**TDI[d]**	**RIVM (Baars et al. 2001)**	**Dietary intake of copper**	**58.8**

Metal/ metalloid	Health based guidance value	Type of Health based guidance value	Health based guidance value set by	Health based guidance value set for	Health based guidance value (mg week^{-1}) [a]
	5 mg day^{-1}	UL[g] of the safe range of mean population intakes	EC SCF (2003)	Dietary intake of copper	35
Iron	**0.8 mg kg bw^{-1} day^{-1}**	**PMTDI[h]**	**JECFA (1983)**	**Iron from all sources, except iron oxides used as coloring agents, supplemental iron taken during pregnancy and lactation and supplemental iron for specific clinical requirements**	**336**
Lead	**25 µg kg bw^{-1} week^{-1}**	**PTWI[b]**	**IPCS (2000)**	**Lead from all food sources**	**1.5**
	0.54 µg kg bw^{-1} day^{-1}	**BMDL$_{01}$[e]**	**EFSA (2010[b])**	**Maternal BMDL$_{01}$ intake level for lead for neurodevelopmental effects on the developing fetus**	**0.23**
Magnesium	**250 mg day^{-1}**	**Tolerable Upper Intake Level**	**EC SCF (2001)**	**Readily dissociable magnesium salts in nutritional supplements, water, or added to food and beverages**	**1750**
Mercury	2 µg kg bw^{-1} day^{-1}	TDI[d]; TDI[d]	ICPS (2003); RIVM (Baars et al. 2001)	Inorganic mercury	0.84
	4 µg kg bw^{-1} week^{-1}	**PTWI[b]; TWI[d]**	**JECFA (2011[b]); EFSA (2012)**	**Inorganic mercury**	**1.68**
Zinc	**500 µg kg bw^{-1} day^{-1}**	**TDI**	**RIVM (Baars et al. 2001)**	**Dietary intake of zinc**	**210**
	0.3-1.0 mg kg bw^{-1} day^{-1}	PMTDI[h]	JECFA (1982)	Dietary intake of zinc	126-420

Notes:
Values in bold were the values used in the present paper for the safety assessments.
[a] expressed on a weekly basis and calculated for a 60 kg adult (except for magnesium and WHO/EFSA health based guidance value for chromium);
[b] Provisional Tolerable Weekly Intake
[c] Tolerable Weekly Intake
[d] Tolerable Daily Intake
[e] Benchmark Dose Lower Confidence Limit of 1% extra risk
[f] Benchmark Dose Lower Confidence Limit of 0.5% extra risk
[g] Upper Limit
[h] Provisional Maximum Tolerable Daily Intake

For our risk assessment of lead from clay products, we initially selected for enforcement purposes the PTWI for lead from all food sources of 25 µg kg bw^{-1} week^{-1}, set by the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) (IPCS 2000). In the course of our study, EFSA concluded in a

Scientific Opinion on Lead in Food that this PTWI, established by IPCS, was no longer appropriate. EFSA based this conclusion on the absence of evidence for a threshold for a number of critical endpoints including developmental neurotoxicity. EFSA also concluded that due to maternal exposure to lead at current dietary exposure levels a risk to the developing fetus could not be excluded. EFSA derived a maternal $BMDL_{01}$ for neurodevelopmental effects (a decrease of 1 IQ point) on the developing fetus of 0.54 µg kg bw^{-1} day^{-1}, which is equivalent to 0.23 mg $week^{-1}$ for a 60 kg adult (EFSA 2010).

For our risk assessment of inorganic arsenic we applied the TDI of 1.0 µg kg bw^{-1} day^{-1}, which was established by RIVM focusing on skin cancer as the critical effect (Baars et al. 2001). This TDI, which we applied for enforcement purposes, is equivalent to 0.42 mg $week^{-1}$ for a 60 kg adult. During our study, EFSA published a Scientific Opinion on Arsenic in Food, in which $BMDL_{01}$ values for cancer in the lungs, skin and bladder and skin lesions ranging from 0.3 to 8 µg kg bw^{-1} day^{-1} were derived (EFSA 2009[a]). Furthermore, JECFA (2011[a]) also published a safety evaluation of arsenic in food, in which JECFA derived a $BMDL_{0.5}$ of 3.0 µg kg bw^{-1} day^{-1} for inorganic arsenic, resulting in a 0.5% increased incidence of lung cancer (JECFA 2011[a]). We applied the lowest BMDL value derived in these recent risk assessments, which is the $BMDL_{01}$ intake value of 0.3 µg kg bw^{-1} day^{-1} derived by EFSA for lung cancer which is equivalent to 0.13 mg $week^{-1}$ for a 60 kg adult, to our data (EFSA 2009[a]). For arsenic no specific risk assessment has been performed for the developmental effects mentioned (stillbirth etc.).

For our risk assessment of mercury in clay products, we assumed that mercury in clay is present as inorganic mercury and we selected therefore a TWI for inorganic mercury of 4 µg kg bw^{-1} day^{-1} which is based on renal toxicity and was recently established by EFSA (EFSA 2012). This TWI is equivalent to 1.68 mg $week^{-1}$ for a 60 kg adult (Table S-3). Data on the reproductive toxicity of inorganic mercury compounds in humans are lacking, but animal studies suggest that exposure to inorganic mercury compounds could result in toxic effects during development (EFSA 2012).

For our risk assessment of aluminum we selected the JECFA-PTWI of 2 mg kg bw^{-1} $week^{-1}$ which applies to all aluminum compounds in food, including food additives and is equivalent to 120 mg $week^{-1}$ for a 60 kg adult (JECFA 2011[b]).

In agreement with European Regulation (EC) No. 333/2007 we established for enforcement purposes for each metal and metalloid the estimated weekly intake level at which the health based guidance value expressed on a weekly basis for a 60 kg adult was exceeded beyond reasonable doubt (EC 2007). We derived these decision limits by adding to the selected health based guidance values for these elements, the analytically obtained expanded measurement uncertainty (Table 1). In the course of our study, the European Commission established maximum levels for lead, mercury, and cadmium in food supplements in Regulation (EC) No. 1881/2006, but no limit has yet been

proposed for arsenic. These limits apply since 1 July 2009 (EC 2008) and we also applied the EC maximum levels to our data. Because of the dose form, the health clays included in the current study, but not the traditional clay products, are covered by the definition of food supplements as specified in the European Directive 2002/46/EC (EC 2002[a]). Therefore, the EC maximum levels for these metals in principle only apply to the health clays investigated in this study. In accordance with the requirements established in Regulation (EC) No. 333/2007, we established for lead, mercury, and cadmium at what level the respective maximum product level in Regulation (EC) No. 1881/2006 is exceeded beyond reasonable doubt (EC 2008). In order to define such decision limits, the analytically obtained expanded measurement uncertainty values for lead, mercury and cadmium were added to these EC maximum product levels (Table 2).

Table 1. Decision limits to the health based guidance values at which health based guidance values are exceeded beyond reasonable doubt, consisting of the health based guidance value plus the expanded measurement uncertainty.

Metal/metalloid	Health based guidance values (mg week^{-1})[a]	Expanded measurement uncertainty (mg week^{-1})[a.b]	Decision limit (to health based guidance values) (mg week^{-1})[a]
Aluminum	120	24[c]	144
Arsenic TDI	0.42	0.10	0.52
Arsenic $BMLD_{01}$	0.13		
Cadmium	0.15	0.017	0.17
Chromium	2100	630[c]	2730
Copper	58.8	6.30[c]	65.1
Iron	336	112[c]	450
Lead PTWI	1.5	0.27	1.77
Lead $BMDL_{01}$	0.23		
Magnesium	1750	177[c]	1930
Mercury	1.68	0.26	1.94
Zinc	210	21.5[c]	232

Notes:

[a] expressed on a weekly basis and calculated for a 60 kg adult (where applicable), derived from Table S-3.

[b] determined by in house validation

[c] recovery not included in measurement uncertainty

Table 2. Decision limits at which EU maximum product levels for lead, mercury and cadmium in food supplements as set in Commission Regulation (EC) No. 1881/2006[a] are exceeded beyond reasonable doubt. consisting of the EU maximum levels plus the expanded measurement uncertainty of the method applied by NVWA.

Metal	EC maximum product levels in food supplements (mg kg^{-1})	Expanded measurement uncertainty [b] (mg kg^{-1})	Decision limits for maximum product levels in food supplements (mg kg^{-1})
Cadmium	1.0	0.114	1.114
Lead	3.0	0.54	3.54
Mercury	0.1	0.016	0.116

Notes:

[a] as amended by Regulation (EC) No. 629/2008

[b] determined by in house validation

Furthermore, we assumed that the metal and metalloid content of the clay products would not be fully available for intestinal absorption and therefore we refined our risk assessment by applying a range of bioaccessible fractions per metal and metalloid in clay to our data. For this we collected data of *in vitro* bioaccessibility studies of metals and the metalloid arsenic in soil fractions (Table S-4). In order to estimate the range of the bioaccessible fractions we selected per metal or metalloid the lowest and highest reported bioaccessibility values which are shown in Table 3. For the refinement of our risk assessment we based our estimated intake levels on three bioaccessibility scenarios. For the first scenario we corrected for the lower bound of the bioaccessible fraction, which presents the least conservative estimated intake estimation. For the second scenario we corrected for the upper bound of bioaccessible fraction, which presents a less conservative estimated intake. In the third scenario no correction for bioaccessibility was made, which means that the measured metal and metalloid content would be fully bioaccessible, thereby presenting the worst case estimated intake level scenario.

Supplementary Table S-4. Bioaccessible fractions of selected metalloid and metals in soil identified in literature.

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
Aluminum	0.26-1.7					
		0.26-1.7 (median)	Soil (ground soil) (n=9), termite nest soil (n=8), traditional herbal soil remedies (n=5)	Uganda	PBET[b]	Smith et al (2000)
		0.53 (mean)	Soil termite nest (n=2)	Zimbabwe	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
		1.3 (mean)	Soil (n=1)	USA (North Carolina)	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
		1.7 (mean)	Soil suburb city (n=3)	China (Hunan province)	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
Arsenic	1-44					
		1-22 (range), 5 (median), 6.9 (average)	highly mineralised locations containing geogenic As sources (gossans) (n=11)	Australian Capital Territory and South Australia	SBET[d]	Juhaz et al. (2007)
		4.8 and 6.3 (mean of 250 µm and 2 mm size fractions)	Surface soils (n=20)	N-S transect United States and Canada	Simulated gastric fluid described by Drexler and Brattin (2007)[e]	Morman et al. (2009)
		5-14 (range), 9 (mean), 9 (median)	Geogenic domains dominated by iron stones (n=49)	Northampton urban area, United Kingdom	UBM[f]	Appleton et al. (2012)
		9 (median - not affected by mining activities)	mineralized soils (n=20)	Devon, UK	PBET[b]	Palumbo-Roe and Klinck (2007)

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
		9.7-28.7 (mean, range for three different ingestion scenarios)	Urban playground soils (n=25)	Urban Uppsala, Sweden	*In vitro* digestion described by Oomen et al. (2002)[g]	Ljung et al. (2007)
		25±19.5 (mean relative bioaccessible fraction across all soils	Different soils collected from different regions (n=50)	Australia	SBET[d]	Smith et al. (2009)
		29	Forest soil with high clay content from abandoned gold mine North Brookfield District (n-=1)	Nova Scotia, Canada	PBET[b]	Meunier et al. (2010)
Cadmium	12.9-68					
		12.9-27.2 (mean, range for three different ingestion scenarios)	Urban playground soils (n=25)	Urban Uppsala, Sweden	*In vitro* digestion described by Oomen et al. (2002)[g]	Ljung et al. (2007)
		16-59 (range), 31 (mean), 32 (median) for gastro-intestinal phase 58-61 (range), 68 (mean) , 68 (median) for gastric phase	Topsoil samples from 2 lead and zinc smelters (n=27)	Northern France	UBM[f]	Roussel et al. (2010)
		58.8 and 64.9 (mean of 2 mm and 250 µm size fractions)	Surface soils (n=20)	N-S transect United States and Canada	Simulated gastric fluid described by Drexler and Brattin (2007)[e]	Morman et al. (2009)
Chromium	1-16					

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
		1-16 (range)	0-10 cm in depth samples from Tirono (n=5) and Sevilla (n=5)	Northern Italy and southern Spain	SBET[d]	Madrid et al. (2008)
		1.4 and 1.6 (mean of 2 mm and 250 µm size fractions)	Surface soils (n=20)	N-S transect United States and Canada	Simulated gastric fluid described by Drexler and Brattin (2007)[d]	Morman et al. (2009)
		1.9-4.5 (mean, range for three different ingestion scenarios)	Urban playground soils (n=25)	Urban Uppsala, Sweden	*In vitro* digestion described by Oomen et al. (2002)[g]	Ljung et al. (2007)
Copper	11.1-60					
		11.1 (median)	Soil (n=13)	Uganda	0.1 M HCl extraction[h]	Abrahams (1997)
		19.7-54.5 (mean)	Soil	Bengali area south Asia	PBET[b]	Abrahams et al (2006)
		38-60 (range)	0-10 cm in depth soil samples from Tirono (n=5) and Sevilla (n=5)	Northern Italy and southern Spain	SBET[d]	Madrid et al. (2008)
Iron	0.1-4.1					
		0.1- 2.9 (median bioaccessibility)	Soil (ground soil (n=9), termite nest soil (n=8), traditional herbal soil remedies (n=5)	Uganda	PBET[b]	Smith et al (2000)
		0.2-4.1	Soil	Bengali area south Asia	PBET[b]	Abrahams et al (2006)
		1.7 (mean)	Soil (n=1)	USA (North Carolina)	Gastrointestinal mineral dissolution[b]	Aufreiter et al. (1997)
		1.7 (median)	Soil (n=13)	Uganda	0.1 M HCl extraction[h]	Abrahams (1997)

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
		2.4 (mean)	Soil suburb city (n=3)	China (Hunan province)	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
Lead	2.8-76					
		2.8-15.4 (mean, range different ingestion scenarios)	Urban playground soils (n=25)	Urban Uppsala, Sweden	*In vitro* digestion described by Oomen et al. (2002)[g]	Ljung et al. (2007)
		3.0 and 3.4 (point estimates)	Soil (n=2)	Bengali area south Asia	PBET[b]	Abrahams et al. (2006)
		4.0 and 18.3 (mean of 2 mm and 250 µm size fractions, respectively)	Surface soils (n=20)	N-S transect United States and Canada	Simulated gastric fluid described by Drexler and Brattin (2007)[e]	Morman et al. (2009)
		14-63 (range), 32 (mean), 32 (median) for gastro-intestinal phase 33-76 (range), 62 (mean), 65 (median) for gastric phase	Topsoil samples from 2 lead and zinc smelters (n=27)	Northern France	UBM[f]	Roussel et al. (2010)
		16.3 (median)	Soil (n=13)	Uganda	0.1 M HCl extraction[h]	Abrahams (1997)
		17.8-59.1 (range) and 42 (median)	soils (n=19)	New South Wales and South Australia, Australia	SBET[d]	Lamb et al. (2009)
		30-60 (range)	0-10 cm in depth soil samples from Torino (n=5) and Sevilla (n=5)	Northern Italy and southern Spain	SBET[d]	Madrid et al. (2008)
Magnesium	5.5-51.7					
		5.5-51.7 (mean)	Soil	Bengali area south Asia	PBET[b]	Abrahams et al (2006)

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
		7-33 (median bioaccessibility)	Soil (ground soil (n=9), termite nest soil (n=8), traditional herbal soil remedies (n=5)	Uganda	PBET[b]	Smith et al (2000)
		9.2 (mean)	Soil suburb city (n=3)	China (Hunan province)	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
		9.5 (mean)	Soil termite nest (n=2)	Zimbabwe	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
		10.8 (mean)	Soil (n=1)	USA (North Carolina)	Gastrointestinal mineral dissolution[c]	Aufreiter et al. (1997)
		28.2 (median)	Soil (n=13)	Uganda	0.1 M HCl extraction[h]	Abrahams (1997)
Mercury	0.3-46					
		0.3-14 (range), 3.2 (average) (n=19)\46 (point estimate (n=1) average for n=20: 5.3	East Fork Poplar Creek, Oak Ridge site (HgS-contaminated) (n=20)	Tennessee, United States	*In vitro* simulation of the stomach and small intestines	Barnett and Turner (2001)
		<0.93-<3.14 (gastric phase) <1.02-<3.15) (intestinal phase)	Clays near a chlor-alkali plant in eastern Canada (n=2), and silty loam collected near a gold mine in Quebec (n=1)	Canada	CDM method described by Barnett and Turner (2001)[i]	Zagury et al. (2009)

Metal or metalloid	Reported range of the bioaccessible fraction (%)[a]	Bioaccessible fraction (%)	Type of soil	Origin soil/clay	Method	Author(s)
		<10	Unpublished *in vitro* study on soil from site in Los Gatos (n=unknown)	California, United States	5-hr 'stomach phase' incubation at a pH of 2.5 followed by a 4-hr 'small intestinal phase' at a pH of 6.5	Schoof and Nielsen (1997)
Zinc	8-85					
		8-47 (range), 23 (mean), 23 (median) for gastro-intestinal phase 17-85 (range), 47 (mean), 48 (median) for gastric phase	Topsoil samples from 2 lead and zinc smelters (n=27)	Northern France	UBM[f]	Roussel et al. (2010)
		9.8-33.3 (mean)	Soil	Bengali area south Asia	PBET[b]	Abrahams et al (2006)
		12.6 (median)	Soil (n=13)	Uganda	0.1 M HCl extraction[h]	Abrahams (1997)
		21-83 (median)	Soil (ground soil (n=9), termite nest soil (n=8), traditional herbal soil remedies (n=5)	Uganda	PBET[b]	Smith et al (2000)
		34-83 (range)	0-10 cm in depth samples from Torino (n=5) and Sevilla (n=5)	Northern Italy and southern Spain	SBET[d]	Madrid et al. (2008)

Notes:

[a] For the estimation of the range of the bioaccessible fraction we selected from the studies, which are shown in this table, the lowest and highest reported bioaccessible value per metal or metalloid.

[b] PBET; *in vitro* physiologically based extraction test (simulates gastric and small intestinal mode of action)

[c] Gastrointestinal mineral dissolution; Low pH chemical extraction

[d] SBET; simplified bioaccessibility extraction test; represents the gastric phase

[e] Simulated gastric fluid described by Drexler and Brattin (2007); simulates gastric fluid

[f] UBM; Unified BARGE Method, which is an *in vitro* physiological gastro-intestinal (GI) simulation described by

Wragg et al. (2011) and Denys et al. (2012); includes gastric and gastro-intestinal phases
[g] *In vitro* digestion described by Oomen et al. (2002); model includes mouth, stomach and small intestines
[h] 0.1 M HCl extraction; simulates stomach
[i] CDM method described by Barnett and Turner (2001); includes gastric phase and intestinal phase extractions

Results

Contents of metals and the metalloid arsenic in clay products

In total 63 samples were analyzed for selected metals and the metalloid arsenic. These 63 samples could be subdivided in the following categories: African clays (n=10), Suriname clays (n=26), and health clays (n=27). Not every sample was analyzed for all selected elements. Table S-2 shows that the highest level of lead (99.7 mg kg^{-1}) was found in a Suriname clay product named 'pimba'. The mean lead content in health clay products was significantly lower ($P<0.05$) than the mean lead content found in traditional clay products of African and Suriname origin. The highest level of arsenic (45.1 mg kg^{-1}) was found in a health clay product, and that of mercury (2.20 mg kg^{-1}) in an African clay product named 'Mabele broken Zaïre'. The highest cadmium levels were found in a health clay product (0.75 mg kg^{-1}), that of aluminum (280,000 mg kg^{-1}) in a clay product originating from Suriname and those for magnesium (63,500 mg kg^{-1}), copper (67 mg kg^{-1}), and chromium (205 mg kg^{-1}) in health clay products. The highest levels for iron (210,000 mg kg^{-1}) and zinc (220 mg kg^{-1}) were found in African clay products.

Estimated weekly intake levels in relation to health based guidance values

From the contents of the metalloid and metals determined in the individual clay products and their estimated or recommended ingestion levels of traditional and health clays, the estimated weekly intake levels were calculated as described in the Material and Methods section for three scenarios: by applying the estimated lower and upper bounds of the reported bioaccessibility ranges, which are shown in Table 3, and by assuming that the contents of all metals and arsenic would be fully bioaccessible.

Table 3. Reported lower and upper bound of bioaccessible fraction per metal or metalloid

Metal/metalloid	Lower bound (%)[a]	Upper bound (%)[a]
Aluminum	0.26	1.7
Arsenic	1.0	44
Cadmium	12.9	68
Chromium	1.0	16
Copper	11.1	60
Iron	0.1	4.1
Lead	2.8	76
Magnesium	5.5	51.7
Mercury	0.3	46
Zinc	8.0	85

Note:

[a] For the estimation of the range of the bioaccessible fraction we selected from the studies which are shown in Table S-4 the lowest and highest reported bioaccessible value per metal or metalloid.

Table S-5 shows for each metal or metalloid in the combined group of clay products and each of the subgroups of clay products the mean, highest and lowest estimated intake levels, resulting from the use of these products at estimated or recommended dose levels, assuming that the metals and the metalloid would be 100% bioaccessible. Table 4 shows for each analyzed metal or metalloid and for the three scenarios the number of clay products for which the use would lead to an exceedance of the health based guidance values. In Table 4 it is shown that, when applying the lower bound of the reported bioaccessibility range, except for the PTWI for aluminum and $BMDL_{01}$ for lead, no individual estimated intake levels of lead and arsenic exceed the health based guidance values. From Table 5 it is shown that the ranges of MOE values for lead in traditional clay products are above 1 except for the full bioaccessibility scenario and upper bound scenario. The ranges of MOE values for arsenic are above 1 except for the African clay products for the full bioaccessibility scenario and the upper bound scenario (Table 5). No clay products when used at the estimated doses were found to result in intake levels exceeding health based guidance values of mercury and cadmium for all three bioaccessiblility scenarios. For mercury, considering that this metal would be 100% bioaccessible, the highest estimated weekly intake level was found in a clay product of African origin (0.65 mg $week^{-1}$). The use of this clay product, at the daily dose level of 42 g, would result in a weekly intake amounting to 39% of the selected health based guidance value of 1.68 mg $week^{-1}$ for mercury (Table S-3). For cadmium, considering this metal would be fully bioaccessible, the highest estimated weekly intake, when used at the estimated daily dose level of 42 g of the clay products analyzed for this metal, was 87% of the selected health based guidance value of 0.15

mg week^{-1} (Table S-3), and was found in a mabele originating from Africa (0.13 mg week^{-1}). At the estimated daily dose level of 42 g, use of a clay product from Suriname would result in an estimated weekly aluminum intake of 83,000 mg week^{-1}. This level exceeds the selected health based guidance value up to 600 times. When considering the full bioaccessibility scenario, or the lower and upper bounds of the bioaccessibility range for aluminum, the use of respectively 53, 6 and 32 clay products at the estimated or recommended dose levels, would lead to intake levels that would exceed the health based guidance value for this metal (Table 4). Assuming that the iron in the clay products is fully accessible, the use of the clay products would result in relatively high intake levels of this metal and could lead to a considerable exceedance of health based guidance levels. However, when taking into account the reported bioaccessibility range of 4.1 to 0.1%, only two clay products or no clay products at all, would lead to an exceedance of the health based guidance value (Table 4). Assuming magnesium would be fully bioaccessible, the use of eight out of the 60 products analyzed for magnesium would lead to exposure above the Tolerable Upper Intake Level, equivalent to 1750 mg week^{-1}. However, this number would be reduced to only three or none at all when considering that the bioaccessibility of the magnesium contents would range between 51.7% and 5.5% (Table 4). Use of the analyzed products would not result in estimated weekly intakes of copper, chromium and zinc above the corresponding health based guidance values even when assuming full bioaccessibility for these metals.

The EU has set maximum levels for lead, cadmium and mercury but these are only applicable to the health clays. Table 6 shows the number of health clay products exceeding the EU maximum levels for food supplements for these metals in comparison to the number of clays of which the use would lead to an exceedance of the health based guidance levels taking into account the three scenarios of bioaccessible fractions.

Table S-5. Metal and metalloid estimated weekly intake levels (mg week^{-1}) in clay products[a]

Metal/metalloid All clays	Number of clay products analyzed for metal (n)	Mean (mg week^{-1})	Lowest value (mg week^{-1})	Highest value (mg week^{-1})
Aluminum	55	26000	< LOQ (n=7)	83000
Arsenic	61	0.914	< LOQ (n=1)	7.99
Cadmium	38	0.0584	< LOQ (n=23)	0.133
Chromium	62	14.9	0.016	56.7
Copper	45	3.2	< LOQ (n=2)	18
Iron	60	3600	43	62000
Lead	62	5.97	< LOQ (n=1)	29.3
Magnesium	60	854	1.28	7310
Mercury	32	0.0714	< LOQ (n=15)	0.646
Zinc	47	8.2	0.13	65

Metal/metalloid All clays	Number of clay products analyzed for metal (n)	Mean (mg week^{-1})	Lowest value (mg week^{-1})	Highest value (mg week^{-1})
African clays				
Aluminum	7	32000	< LOQ (n=3)	45000
Arsenic	10	2.31	0.600	5.10
Cadmium	8	0.0869	< LOQ (n=2)	0.133
Chromium	10	27.5	14	37.6
Copper	8	8.1	3.7	18
Iron	10	11000	2000	62000
Lead	10	10.1	5.64	14.0
Magnesium	10	1780	274	5460
Mercury	8	0.149	0.0190	0.646
Zinc	8	23	10	65
Suriname clays				
Aluminum	24	48000	< LOQ (n=1)	83000
Arsenic	25	0.745	0.0932	7.99
Cadmium	17	0.0718	< LOQ (n=7)	0.119
Chromium	25	22.7	1.46	56.7
Copper	13	3.3	0.76	12
Iron	25	2200	790	12000
Lead	25	10.0	< LOQ (n=1)	29.3
Magnesium	25	333	13.9	2650
Mercury	12	0.0817	< LOQ (n=1)	0.535
Zinc	13	6.5	1.7	13
Health clays				
Aluminum	24	2700	< LOQ (n=3)	12000
Arsenic	26	0.541	< LOQ (n=1)	1.70
Cadmium	13	0.0233	< LOQ (n=14)	0.630
Chromium	27	3.12	0.0160	12.5
Copper	24	1.51	< LOQ (n=3)	5.5
Iron	25	1800	43	7100
Lead	27	0.697	0.0250	2.70
Magnesium	25	1010	1.28	7310
Mercury	12	0.00941	< LOQ (n=14)	0.0400
Zinc	26	4.6	0.13	17

Note: [a] not corrected for the bioaccessible fractions of the metals and metalloid

Table 4. Number of clay products the use of which would lead to estimated intakes exceeding selected health based guidance values[a], assuming either 100% bioaccessible or taking into account the reported upper and lower bound values of the bioaccessible fraction.

Metal/ metalloid	No. of products tested per metal/metalloid	100% bioaccessible	Lower bound	Upper bound
Aluminum	55 (7/24/24)[b]	53 (7/24/22)[c]	6 (0/6/0)[c]	32 (7/23/2)[c]
Arsenic TDI	61 (10/25/26)[b]	26 (10/5/11)[c]	0	13 (7/3/3)[c]
Arsenic $BMDL_{01}$		49 (10/22/17)[c]	0	32 (10/8/14)[c]
Cadmium	38 (8/17/13)[b]	0	0	0
Chromium	62 (10/25/27)[b]	0	0	0
Copper	45 (8/13/24)[b]	0	0	0
Iron	60 (10/25/25)[b]	56 (10/25/21)[c]	0	2 (1/1/0)[c]
Lead PTWI	62 (10/25/27)[b]	36 (10/24/2)[c]	0	35 (10/24/1)[c]
Lead $BMDL_{01}$		51 (10/25/16)[c]	21 (6/15/0)[c]	50 (10/25/15)[c]
Magnesium	60 (10/25/25)[b]	8 (3/2/3)[c]	0	3 (2/0/1)[c]
Mercury	32 (8/12/12)[b]	0	0	0
Zinc	47 (8/13/26)[b]	0	0	0

Notes:

[a] Expressed on a weekly basis and calculated for a 60 kg adult (Table S-3)

[b] Between brackets respective numbers of African, Suriname and health clay products tested per metal or metalloid

[c] Between brackets respective numbers of African, Suriname and health clay products exceeding the health based guidance values

Table 5. Ranges in Margins of Exposure (MOE) for lead[a] and arsenic[b] in three categories of clay products for three scenarios: lead in clay products is either 100% bioaccessible, or the bioaccessability is at the reported upper bound level, or at the reported lower bound level.

Product	100% bioaccessible		Upper bound		Lower bound	
	Lead	Arsenic	Lead	Arsenic	Lead	Arsenic
African clays (n= 10)	0.02-0.04	0.03-0.22	0.02-0.05	0.06-0.49	0.59-1.46	2.6-21.7
Suriname clays (n=25)	0.01-0.44	0.02-1.4	0.01-0.58	0.04-3.2	0.28-15.8	1.6-139.5
Health clays (n=27)	0.09-9.20	0.08-5.5	0.11-12.1	0.17-12.6	3.04-328.6	7.7-553.2

Notes:

[a] expressed as the ratio between the maternal $BMDL_{01}$ intake level for lead for neurodevelopmental effects on the developing fetus of 0.54 µg kg bw^{-1} day^{-1} (Table S-3) (EFSA 2010[b]) and the intake levels for lead in clay products, expressed per kg bw^{-1} day^{-1}.

[b] expressed as the ratio between the $BMDL_{01}$ values for use in the risk characterization for inorganic arsenic for cancer in the lungs of 0.3 µg kg bw^{-1} day^{-1} (Table S-3) (EFSA 2009[a]) and the intake levels for arsenic in clay products, expressed per kg bw^{-1} day^{-1}.

Table 6. Number of health clay products (n=27) exceeding the decision limits based on the EU maximum levels for food supplements and health based guidance values assuming 100% bioaccessible or corrected for the reported upper and lower bounds of the bioaccessible fractions.

Metal	EU maximum levels[a]	health based guidance value, 100% bioaccessible[b]	health based guidance value[b] upper bound of the bioaccessible fraction	health based guidance value[b] lower bound of the bioaccessible fraction
Cadmium	-	-	-	-
Lead PTWI	24	2	1	-
Lead $BMDL_{01}$	24	16	15	-
Mercury	4	-	-	-

Notes:

[a] taking into account the expanded measurement uncertainty

[b] expressed on a weekly basis and calculated for a 60 kg adult

Discussion

In this study different metals and the metalloid arsenic were quantified in traditional and health clay products available on the Dutch market. Of special interest were, relative to typical food stuff concentrations, high lead, arsenic and aluminum contents found in these clay products.

Dean et al. (2004) reported levels for chromium and lead in calabash chalk, quantified using Energy Dispersive X-ray Fluorescence (EDXRF), which were comparable to the levels found in African clay products in our study. In the study of Dean et al. (2004), arsenic, mercury and cadmium were not detected. However, Ibeanu et al. (1997) found lead, by using EDXRF, in three samples of Nigerian and Kenyan clays at levels that were two to three times higher than the mean level found in the African clay products in the current study. In the UK, the FSA (2002) reported for calabash chalk, lead levels of 8.2 up to 16.1 mg kg^{-1}, which were below the range of lead levels of African clay products included in our study (19.2– 47.6 mg kg^{-1}). Katulek et al. (2010) published data similar to data from our study on lead and mercury contents of 88 African clay products analyzed with Atomic Absorption Spectrophotometry. The mean cadmium value of the samples determined by Katulek et al. was four times lower than in our study for the African clay products. Using the same type of analysis (ICP-MS) as we did, Al-Rmalli et al. (2010) published data for lead, arsenic, cadmium, iron and zinc in sikor, obtained in ethnic Bangladeshi shops in the United Kingdom, which were similar to data from our study.

Our analytical method was not equipped to determine the speciation of arsenic and metals in the clay samples, and the results were thus expressed as the total amount of the metalloid or metals. Moreover, except for arsenic (see below), data in literature on the speciation of metals in different types of clays and soils were generally

scarce and difficult to relate to the samples in the present study because the geological identity of the clay products in our study was not established. Speciation is important regarding differences in toxicity. An additional factor to take into account when performing a risk assessment is the bioaccessibility of metals and metalloids present in clay products. To this end we identified in the literature bioaccessibility studies for metals and arsenic from soil including clay (Table S-4). In these studies different bioaccessiblility models and geological soil types were used. It should be noted that the 0.1 M HCl extraction method (Abrahams 1997), the simulated gastric fluid method described by Drexler and Brattin (2007) and Morman et al. (2009) and the simplified bioaccessibility extraction test (SBET) (Madrid et al. 2008; Lamb et al. 2009; Smith et al. 2009) only include simulation of the stomach at a low pH and do not include a change in pH when passing to the small intestine. Chemical elements such as lead are solubilised in the acidic human stomach but will subsequently precipitate or adsorb onto solid particles at the higher intestinal pH (Abrahams 2012). Therefore, the bioaccessible fractions calculated from such models which only simulate the stomach are probably overestimated. Another factor which we did not include was the influence of the presence of food on the bioaccessibility (Abrahams 2012). Preferably, bioaccessibility data were taken from naturally enriched (geogenic) soils. Table S-4 includes also studies with geophagic soils originating from Africa and Asia (Abrahams 1997; Aufreiter et al. 1997; Smith et al. 2000; Abrahams et al. 2006). For mercury and cadmium, studies of bioaccessible fractions in uncontaminated soils were scarce. Therefore, for these metals estimations from the selected studies on bioaccessiblility do probably not reflect the real situation for bioaccesssibility of these metals in clay products.

When applying the $BMDL_{01}$ values to the estimated bioaccessibility scenarios for lead the use of many clay products at estimated or recommended dose levels would be without any margin of exposure and in most cases would lead to an exceedance of these $BMDL_{01}$ values. It should be noted that even at the lower bound of the estimated bioaccessibility for lead 21 out of 62 clay products would lead to an exceedance of the $BMDL_{01}$ value for lead (Table 4). To conclude, the offspring of women who use African and Surinam clay products, particularly during pregnancy, may be at risk for retardation in the mental development resulting from lead exposure considering all bioaccessiblility scenarios for lead. Furthermore, it was interesting to note that estimated mean lead intake levels at estimated dose levels for African and Suriname clay products were at least 14 times higher than estimated mean lead intake levels for health clay products when considering the full bioaccessibility scenario (Table S-5).

Arsenic is reported to be mainly present in soils as inorganic arsenic, in the form of arsenate (As(V)) and arsenite (As(III)) (Lin and Puls 2000; Turpeinen et al. 2003; Wang and Mulligan 2008). In sikor, the main species was inorganic arsenic (As(V)), which was

found in levels of up to 100% of the total extractable arsenic (Al-Rmalli et al. 2010). These arsenic species have a considerably higher toxicity than organic arsenic species found in e.g. seafood (EFSA 2009[a]). The use of respectively 49 and 32 out of 61 clay products would lead to an exceedance of the $BMDL_{01}$ for the scenarios that all the arsenic from clay products would be fully bioaccessible and for the estimated upper bound of the bioaccessible fraction (Table 4). It can therefore be concluded that women who use these clay products during pregnancy at the estimated dose levels could be exposed to levels of inorganic arsenic that could be of concern.

For mercury the use of clay products at the estimated dose levels amounts to exposure levels of 39%, 18% and 0.1% of the selected TWI for mercury for the full bioaccessibility scenario, the estimated upper bound and lower bound of the bioaccessible fraction respectively. To conclude, because of the estimated intake levels and the possible limited bioaccessibility of mercury, the associated health risks could be limited.

Aluminum is a common element in the earth's crust and is present in numerous mineral and organic complexes. Especially in clay, aluminum is present in layered aluminum silicates (Barabasz et al. 2002; Priest 2004; Mol and Spijker 2007; RIVM-RIKILT 2009). It is reported that aluminum is able to reach the placenta and fetus and toxicological effects of this metal include adverse effects on neurodevelopment, growth retardation, skeletal anomalies, embryo toxicity and reduced body weight gain (JECFA 2007; EFSA 2008). High intake levels of aluminum, resulting from use at the estimated dose levels, were found especially for clay products of Suriname origin. However, even when taking into account the possibly limited bioaccessibility of aluminum in soils, which was reported to be in the range of 0.26-1.7%, the use of respectively 6 and 32 clay products of which 6 and 23 clay products were from Suriname would still lead to an exceedance of the health based guidance value for aluminum. This implies a significant health risk.

For magnesium, the Tolerable Upper Intake Level would be exceeded for 8 clay products with a maximum of 4 times the Tolerable Upper Intake Level when assuming magnesium in clay would be fully bioaccessible. Due to the lack of data on the toxicological effects of long term use of magnesium and a broad bioaccessibility range (5.5 - 51.7%), it is difficult to draw conclusions regarding the safety of the magnesium levels in clay products.

Harvey et al. (2000) concluded that the consumption of iron from soil does not significantly influence the iron status of the users in developing countries. The literature shows relatively low bioaccessible fractions for iron in the range 0.1 – 4.1%. For this reason we concluded that adverse effects due to intake of iron are limited.

The estimated intake levels of chromium, copper, and zinc, resulting from use of the clay products at the estimated dose levels, do not exceed the health based

guidance values (Table 4) for the respective metals and therefore no short- or long-term adverse effects are expected. Estimated intake levels of cadmium did not exceed the selected health based guidance value for cadmium either, but a sample was found for which the use at the estimated dose level would result in an intake level of cadmium equivalent to 87% of the selected TWI for cadmium. Considering the relatively high background exposure of cadmium (EFSA 2009[b]) the additional intake level of cadmium by clay products is undesirable.

Table 6 shows the implications of applying the maximum levels for lead, mercury, and cadmium in food supplements which are laid down in Regulation (EC) No. 1881/2006. In comparison with the application of the legal requirement laid down in article 14.1 of Regulation (EC) No. 178/2002 to health clay products (EC 2002[b]), relatively more clay products are non-compliant with the maximum levels for lead and mercury in food supplements.

Overall, the results of the present study reveal that lead and arsenic concentrations in clay products on the Dutch market, when used at the estimated dose levels, may result in estimated intake levels above established health based guidance values. This is true for the reported upper bounds of the bioaccessible fractions of lead and arsenic. Therefore, adverse effects on health of lead and arsenic intake due to the consumption of clay products cannot be excluded. Furthermore, it would be helpful to further quantify the bioaccessibility, in particular for lead, arsenic, mercury, aluminum and cadmium from clay products, e.g. by simulation of their release under the physicochemical conditions in the human gastrointestinal tract. Finally, it would be of interest, given the high levels of clay intake (approximately 40 g per day), to consider possible nutrient interactions, although this remains a topic for future research. It is concluded that consumption of some of the examined types of clay are a cause of concern and use of these clay products should be discouraged.

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CHAPTER 3

Dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-furans) in traditional clay products used during pregnancy

Based on:

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Dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-furans) in traditional clay products used during pregnancy

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Abstract

Geophagy, the practice of consuming clay or soil, is encountered among pregnant women in Africa, Eastern Asia and Latin America, but also in Western societies. However, certain types of clay are known to contain high concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs). The aim of this study was to determine the PCDD/F contents of orally consumed clays purchased from Dutch and African markets. Congener patterns were compared with those of pooled human milk samples collected in eight African countries, to investigate a possible relationship with clay consumption. From the Dutch market thirteen clay products were examined, seven of African and six of Suriname origin. From seven African countries, twenty clay products were collected. All 33 clay products were screened with a cell-based bioassay and those showing a high response were analyzed by GC/HRMS. High PCDD/F concentrations were measured in three clay products from the Dutch market, ranging from 66 to 103 pg TEQ g^{-1}, whereas clay products from African countries were from 24 to 75 pg TEQ g^{-1}. Patterns and relatively high concentrations of PCDD/Fs in human milk samples from the Democratic Republic of the Congo and Côte d'Ivoire suggest a relationship with the consumption of contaminated clay. Frequent use of PCDD/F contaminated clay products during pregnancy may result in increased exposure of the mother and subsequently the developing fetus and new-born child. The use of these contaminated clays during pregnancy should be carefully considered or even discouraged.

Introduction

Soil or clay contaminated with polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and/or dioxin-like PCBs (dl-PCBs) is a known source for these contaminants in the food chain. In 1998 the use of ball clay for feed production in the United States of America (USA) caused the contamination of chickens and catfish with PCDD/Fs (Rappe et al. 1998; Hayward et al. 1999; Ferrario et al. 2000). A similar application of kaolinic clay in Europe, used for the homogeneous mixing of vitamins and minerals in feed, also was shown to be an important source of PCDD/Fs (Jobst and Aldag 2000). In 2004, a French fries company in the Netherlands started to use kaolinic clay for the sorting of potatoes. A by-product, small potato peels, contaminated with the clay, were fed to dairy cows, causing PCDD/F levels as high as 20 pg TEQ (Toxic Equivalents) g^{-1} fat in the milk. The absorption and carry-over of PCDD/Fs from the clay to cow milk appeared to be very efficient (Hoogenboom et al. 2010). The clay used in this process contained PCDD/F concentrations between 1 and 2 ng TEQ g^{-1} product.

Less well-known in Western societies is the intentional consumption of soil and clay materials by humans. This practice called geophagy, has already been described by Aristotle (Wilson 2003), Dioscorides and Avicenna in 40 BC and 1000 AD respectively (Dominy et al. 2007) and subsequently by anthropologists, colonial physicians and explorers like von Humboldt and Livingstone in South America and Africa respectively (Woywodt 2002). In traditional medicine, pregnant women consume clay as a cure against morning sickness but possibly also as a source of minerals like iron (Abrahams and Parsons 1996). Whether this practice has actually beneficial effects has never been described. In Africa, clays for oral use are collected in rural mines, dry river beds, termite nests and walls of housing but they can also be purchased at local markets, traditional remedy shops or even upmarket retail shops. In Suriname, Pimba, which are balls of white or reddish clay, are eaten by some pregnant women of the Creole society. It is used to prevent miscarriage, to prevent acid reflux or to promote delivery but also for spiritual reasons for which it is appliedto the skin (De Korte 2006). Migrants from Africa, Asia and Suriname have introduced the practice of geophagy to Western societies like the United Kingdom (UK), the Netherlands, Belgium and Austria (Mahaney et al. 2000; Abrahams et al. 2006; De Korte 2006; Katulek et al. 2010). These clay products can be purchased in ethnic shops. Data from literature suggests that intake levels are typically between 30 to 80 g day^{-1} (Table S-1).

Table S-1. Examples of estimated dose levels of traditional clay products identified in literature.

Product	Daily dose (range) (g day^{-1})	Study details	Authors
Bentonite clay (Aleppo belouneh)	26 (-)	Market survey in Syria	Bakraji and Karajou (2003)
Eye clay	30 (mean) (1-300)	Interviews Ewe women (n=1248), Ghana	Vermeer (1971)
Soil material	30 (-)	Interviews women Akha tribe in North Thailand	Abrahams and Parsons (1997)
Different clays (riverine, termite and ant clays)	- (28-85)	Field observations five countries southern Africa	Hunter (1993)
Different clays (sedimental red roamy sand (walls houses), grey clay, soft stones (riverbeds), termite clays)	41.5 (median) (3-219)	Interviews pregnant women (n=52) and traditional healers (n=4), Kilifi, Kenya	Geissler et al. (1999)
Sikor	- (49–65)	Interviews pregnant women, Bengali community in the UK	Abrahams et al. (2006)

However, geophagy is considered as an aberrant behavior, and potentially hazardous because of the contamination with toxic constituents such as metals, metalloids, parasites, pathogens, or persistent organic pollutants such as PCDD/Fs (Abrahams 2002). Elevated levels of metals and metalloids such as lead and arsenic were reported in traditional clay products sampled in the UK by the British Food Standards Agency (FSA 2002) and by Kutalek et al. (2010) in traditional clay products from African countries but also from Austria and Belgium. In 2004, the Netherlands Food and Consumer Product Safety Authority (NVWA) started a survey and obtained similar results (RIVM-RIKILT 2009). Knowing the problems caused by PCDD/Fs in clay materials used in feed and food production, these pregnancy clays were also analyzed for the presence of PCDD/Fs. The present paper shows that some of the clay products were indeed highly contaminated. Clay products were also collected in Africa and in some cases also showed high levels of PCDD/Fs.

It is of particular interest to evaluate the possible consequences of consumption of clays with elevated levels of PCDD/Fs. To obtain a first indication, congener patterns of PCDD/Fs in clay products were compared with those in mother milk samples, collected in different African countries within the World Health Organization (WHO) program on human milk (Malisch et al. 2010; UNEP 2013). In addition, a risk assessment was performed on the health risk associated with the intake of clay products with the higher PCDD/F levels observed in this study.

Material and methods

For this study, NVWA inspectors sampled 13 clay products originating from African countries and Suriname at local ethnic shops in the Netherlands. Additionally, 20 different clay products were collected in trading centers in various African countries, namely Uganda (12), Kenya (2), Tanzania (1), Nigeria (1), Mali (1), Côte d'Ivoire (1) and Zimbabwe (2) by African students from Wageningen University. Within the fifth round of the mothers' milk survey, which was organized by the WHO jointly with the United Nations Environment Programme (UNEP), pooled breast milk samples consisting of 50 individual milk samples, were analyzed for PCDD/Fs and dl-PCBs. Milk samples from Africa were collected in Côte d'Ivoire, Democratic Republic of the Congo, Nigeria, Senegal, Ghana, Uganda, Kenya and Mali (WHO 2007; UNEP 2013).

Screening of clays with a cell-based bioassay

A cell-based bioassay (DR CALUX®, Biodetection Systems, the Netherlands) was used by both NVWA and RIKILT to screen the clay products sampled in respectively the Netherlands and African countries for the presence of dioxin-like activity. In this assay, substances binding to the Ah-receptor are detected, which is an essential step in determining the possible presence of PCDD/Fs and dl-PCBs. For this assay 5 g homogenized clay was mixed with 15 ml water/methanol 15/85 (v/v) and subsequently extracted twice with 20 ml hexane/diethyl ether 97/3 (v/v). The combined extracts were reduced to a small volume and purified on a column containing 10 g acid silica (33%), eluted with 40 ml hexane/diethyl ether 97/3 (v/v). The eluate was evaporated using 30 or 40 µl DMSO as a keeper. This was subsequently mixed with 2 ml incubation medium and 0.25 ml of this mixture was added to cells grown in 48-well plates in 0.25 ml medium for two days. After 24 h the medium was removed, the cells washed with phosphate buffer saline (PBS) and lysed. The luciferase activity was determined with a Luminoskan Ascent (Thermo Scientific, Breda, the Netherlands). The luciferase activity for various concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (1 pM to 1 nM) was plotted in a calibration curve. At RIKILT, each test series also included a number of reference feed samples with known PCDD/F and dl-PCB content. This resulted in an estimated level expressed in BEQs (bioequivalents). For screening of clays in the present study, the method was used with a decision limit of 1 pg BEQ g^{-1} product, meaning that samples exceeding this estimated level were analyzed by GC/HRMS.

GC/HRMS

The analysis by GC/HRMS was performed by RIKILT for confirmation of the presence of PCDD/Fs and dl-PCBs in the suspected clay products and performed as described previously (Tuinstra et al. 1994; Hoogenboom et al. 2010). Samples were spiked with ^{13}C labeled standards and extracted using pressurized extraction (ASE, Dionex, Amsterdam, the Netherlands). This was followed by clean-up on an automated system (PowerPrep™ system, FMS, Watertown, USA), which was composed of disposable pre-packed columns (Jumbo silica, mixed bed silica, alumina and carbon (FMS)). This resulted in two fractions, one with mono-ortho and indicator PCBs and the other with PCDD/Fs and non-ortho PCBs. The fractions were concentrated (Turbo-Vap, Buchi, Germany) and analyzed by gas chromatography combined with high resolution mass spectrometry (GC/HRMS) using an Agilent (Wilmington, USA) gas chromatograph 6890N (GC column DB5 MS 60m, 0.25mm i.d., 0.25µm; J&W, Folson, USA) and an AutoSpec Ultima high resolution mass spectrometer (Waters, Milford, USA) operated in electron impact ionization mode using selected-ion monitoring and controlled by a Masslynx data system. GC/HRMS data were processed using DIOXNOP software to determine the concentrations. Subsequently the TEQs were calculated based on WHO TEFs of 1998 (Van den Berg et al. 1998). Levels of non-detectable congeners were set at the Limit of Quantification (upperbound principle). Blanks were included in each test series starting at the ASE extractions and quality control was according to EPA method 1613, revision B. Recoveries were within the range 60-120% except for PCBs 77 and 81, and in some cases OCDD and OCDF. However, in these samples these congeners did not contribute significantly to the TEQ-level, making this deviation acceptable.

2.3 Protocol of the WHO/UNEP coordinated studies

Samples were collected by the participating countries following their national protocols, which dealt primarily with number and type of samples, selection of donors, collection, storage and pooling of samples, and shipping of samples to the reference laboratory. Milk from well-defined groups of 50 mothers was collected and pooled. For selection of donating mothers the following criteria were applied: a) they should be primiparae, b) healthy, c) exclusively breastfeeding one child (i.e. no twins), and d) residing in the area for about five years. For further details see the WHO Website (WHO 2007). Samples of the fifth round were collected in 2008-2009.

Analysis of human milk samples

Analysis of POPs in human milk were performed by the State Institute for Chemical and Veterinary Analysis of Food (CVUA) in Freiburg, Germany (WHO 2000). The analytical procedure for solvent extraction, clean-up and determination by GC-HRMS of the PCDDs/PCDFs, dioxin-like PCBs (DL-PCBs) and non-dioxin-like PCBs was performed as described previously (Malisch et al. 2000; Malisch and van Leeuwen 2002; Malisch and Dilara 2007). The limit of quantification (LOQ) for PCDDs, PCDFs and DL-PCBs expressed as WHO toxic equivalencies (TEQs) was 0.1 pg g^{-1} lipid. As for the clay, TEFs 1998 were applied and levels of non-detectable congeners were set at the Limit of Quantification (upperbound principle). A rigid quality control programme is carried out on a continuous basis as described (Malisch and van Leeuwen 2003; UNEP 2013).

Results

In 2007 and 2009, the NVWA collected 13 clay products, seven termed Mabele (originating from Africa) and 6 termed Pimba (originating from Suriname). These products were screened for PCDD/Fs and dl-PCBs using the DR CALUX® bioassay. Four clay products, showing a response indicative of a level above 1 pg TEQ g^{-1}, were classified as suspected and analyzed by GC/HRMS. Clays termed Mabele salé, Mabele Cameroon 2 and Mabele broken Zaire showed the highest WHO-PCDD/F TEQ levels, being respectively 103.0, 66.3 and 65.5 pg TEQ g^{-1} product (Table S-2).

Table S-2. PCDD/F levels (ng kg^{-1}) in clay products purchased in the Netherlands, Zimbabwe, Nigeria and Côte d'Ivoire (result of a single analysis).

PCDD/F[a] levels (ng kg^{-1})	Mabele salé[b]	Mabele Cameroon 1[b]	Mabele Cameroon 2[b]	Mabele broken Zaire[b]	Nzu Nigeria[c]	Termite nest Zimbabwe[c]	Kaolin Côte d'Ivoire[c]
2,3,7,8-TCDF	<0.05	<0.05	<0.05	<0.05	0.36	<0.05	<0.05
1,2,3,7,8-PeCDF	<0.05	<0.05	<0.05	0.06	0.17	<0.05	<0.05
2,3,4,7,8-PeCDF	0.09	<0.05	<0.05	<0.05	0.12	<0.05	<0.05
1,2,3,4,7,8-HxCDF	0.06	<0.05	<0.05	<0.05	0.07	<0.05	<0.05
1,2,3,6,7,8-HxCDF	0.21	<0.05	0.13	0.17	0.06	<0.05	<0.05
2,3,4,6,7,8-HxCDF	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
1,2,3,7,8,9-HxCDF	<0.05	<0.05	<0.05	0.06	<0.05	0.16	<0.05
1,2,3,4,6,7,8-HpCDF	0.21	<0.05	<0.05	<0.05	0.07	0.05	<0.05
1,2,3,4,7,8,9-HpCDF	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
OCDF	<0.10	<0.10	<0.10	<0.10	<0.10	0.15	<0.10

PCDD/F[a] levels (ng kg^{-1})	Mabele salé[b]	Mabele Cameroon 1[b]	Mabele Cameroon 2[b]	Mabele broken Zaire[b]	Nzu Nigeria[c]	Termite nest Zimbabwe[c]	Kaolin Côte d'Ivoire[c]
2,3,7,8-TCDD	6.1	0.11	6.9	2.9	0.4	<0.05	4.8
1,2,3,7,8-PeCDD	3.4	0.98	2.3	28.6	8.1	0.16	37.5
1,2,3,4,7,8-HxCDD	3.3	6.5	2.2	70.6	35.5	0.29	83.0
1,2,3,6,7,8-HxCDD	31	3.8	19	45	20.2	0.36	42.1
1,2,3,7,8,9-HxCDD	674	12	410	180	49.0	0.66	153.0
1,2,3,4,6,7,8-HpCDD	2051	108	1279	434	480	58	499
OCDD	17438	300	10967	510	1673	7241	419
TEQ (WHO 1998)	103.0	4.5	66.3	65.5	24.1	1.7	75.2

[a] Abbreviations: tetra (T), penta (Pe), hexa (Hx), hepta (Hp) or octa (O) chlorinated dibenzofuran (CDF) or dibenzo-p-dioxin (CDD)

[b] Clay product purchased in the Netherlands

[c] Clay product collected in Africa

Twenty clay products were collected from African countries in 2009 and were all tested at RIKILT with the DR CALUX® bioassay. Figure 1 shows the response of these samples in one test series. Four samples showed a response indicative of a level higher than 1 pg TEQ g^{-1} and were classified as suspected. Three of these samples (S/N13, S/N17 and S/N20) showed a clearly elevated response and one (S/N9) a slightly elevated response. These products originated from Nigeria (Nzu, S/N13), Zimbabwe (termite nest, S/N17), Côte d'Ivoire (Kaolin, S/N20) and Uganda (S/N9). Based on the comparison of the response in the bioassay with those of reference feed samples, the levels of the samples S/N13, S/N17 and S/N20 were estimated to be 13, 11 and 24 pg BEQ g^{-1} product respectively. However, these values are only a rough estimate, also due to the very high response of the samples in the upper part of the calibration curve where the response reaches a plateau.

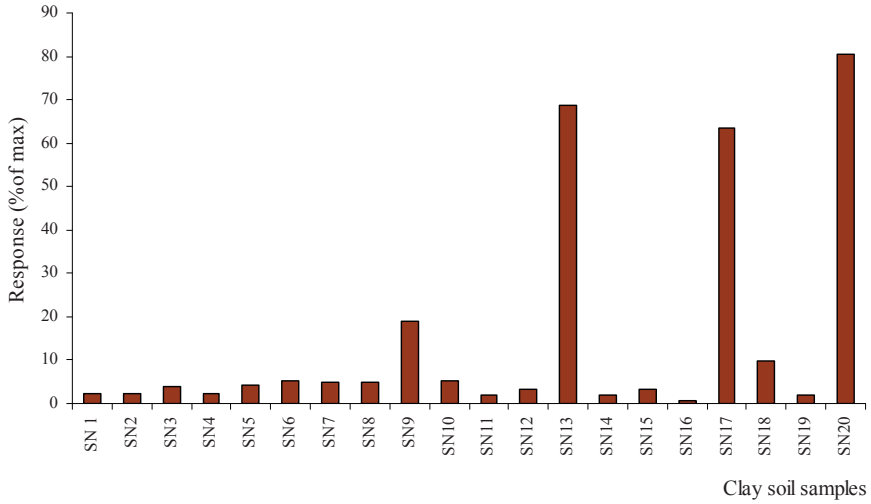

Figure 1. Response of the 20 African clay products in the CALUX assay. Results are expressed as percentage of the maximum response observed with TCDD. Samples with elevated response came from Uganda (S/N9), Nigeria (Nzu, S/N13), Zimbabwe (termite nest, S/N17) and Côte d'Ivoire (Kaolin, S/N20).

Subsequent analysis of the three samples with a high response by GC/HRMS showed PCDD/F levels of respectively 24.1, 1.7 and 75.2 pg TEQ g^{-1} product (Table S-2). Sample S/N9 from Uganda, with a slightly elevated response, and two negative samples (Mabele Nigeria sampled in the Netherlands and S/N16 (Mali)) were also analyzed by GC/HRMS and showed dioxin TEQ levels of 0.3, 0.6 and 0.2 pg TEQ g^{-1} (data not shown). Levels of dl-PCBs were low in all suspected clay samples analyzed by GC/HRMS (4 from the Netherlands and 4 from African countries), being respectively 0.2 and 0.3 pg TEQ g^{-1} in Mabele sale and Mabele Cameroon 1, and less than 0.1 for Mabele Cameroon 2 and Mabele DR Congo for the Dutch samples, and for four of the remaining African samples. Levels of the so-called indicator or non-dioxin-like PCBs (PCBs 28, 52, 101, 138, 153 and 180) were below 1 ng g^{-1} for these eight samples, with the exception of the sample named Mabele Cameroon 1 with also the highest level of dl-PCBs, showing a level for non-dioxin-like PCBs of 9.5 ng g^{-1}.

PCDD/F patterns in selected clay products

Figure 2 shows the congener patterns of PCDD/Fs for the four clay products with the highest levels, expressed as the contribution to the PCDD/F-TEQ level. The TEFs of 1998 were used for this evaluation since these are still applied for official control in Europe until 2012. In general this does not differ very much from applying the TEFs 2005 due to the fact that the TEFs for the PCDDs were not changed with the exception of that for OCDD which was raised three-fold (Van den Berg et al. 1998; 2006). In all these four samples, the PCDFs did not really contribute to the TEQ level. As shown in Table S-2, all clay products showed relatively high levels of HpCDD and octachlorodibenzodioxin (OCDD). However, HpCDD and especially OCDD have a relatively low toxic potency, as reflected by their TEFs 1998 of 0.01 and 0.0001, and as such contribute relatively little to the TEQ level. An exception was the sample from Zimbabwe (S/N17), where OCDD and HpCDD made up 75% of the TEQ, but this sample had a relatively low TEQ-level. Only in this sample the TEQ level would significantly increase (1.8x) by applying the TEFs 2005. Also remarkable are the relatively high levels of 1,2,3,7,8,9-hexachlorodibenzo-p-dioxins (HxCDD), which in some of the Mabele samples showed the highest contribution to the TEQ-level (>60%). In four other samples the contribution of this congener was between 20-25%. In these samples PeCDD and the two other HxCDDs also contributed highly to the TEQ-level.

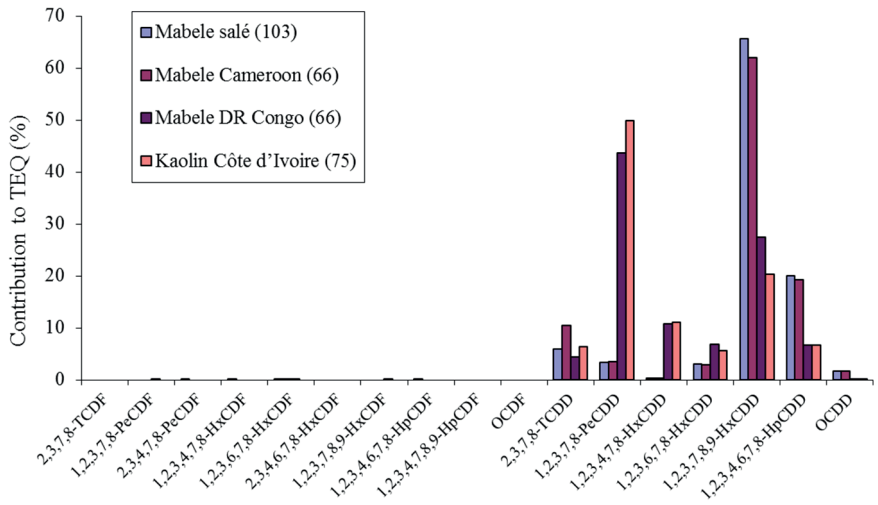

Figure 2. PCDD/F patterns in a number of clay samples collected in The Netherlands (Mabele clays) and Côte d'Ivoire. Patterns are presented in terms of contribution to the TEQ (TEQ in parenthesis in the legend in pg TEQ g^{-1} product).

Comparison of congener patterns in clay and human milk

The WHO has a program to evaluate the levels of PCDD/Fs and dl-PCBs in pooled human milk from various countries in the world (WHO 2007). The last round (2008-2009) included human milk samples from a number of African countries (Malisch et al. 2010). Highest levels of PCDD/Fs were observed in milk samples from Côte d'Ivoire and the Democratic Republic of the Congo, being 11.1 and 12.5 pg TEQ g^{-1} fat respectively. The milk sample from Senegal showed a level of 7.2 pg TEQ g^{-1} fat, but those from the other countries were much lower, being in the range from 1.5 to 3.9 pg TEQ g^{-1} fat (UNEP 2013). Figure 3 shows the patterns observed in the human milk, expressed as the relative contribution of different PCDD/Fs to the TEQ. It is clear that the PCDDs contribute most, varying from 57% for Nigeria to 85 and 90% for Côte d'Ivoire and the Democratic Republic of the Congo. In most samples, also PCDFs and especially 2,3,4,7,8-PeCDF contribute to some extent to the TEQ, but especially in the higher contaminated samples, this contribution is less than 15%. Regarding the relatively high levels and the high contribution of PCDDs, the latter two samples were evaluated in more detail and the patterns compared to those observed in the different clays.

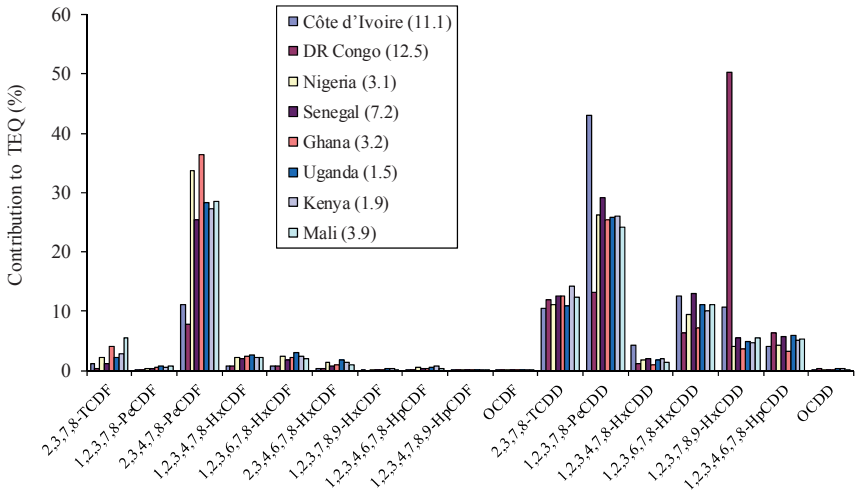

Figure 3. Congener patterns observed in different African human milk samples, expressed as contribution to the PCDD/Fs TEQ (TEQ levels in parenthesis in the legend in pg TEQ g^{-1} fat).

Figure 4A shows the pattern for human milk from the Democratic Republic of the Congo with three different clays that were characterized by an unusually high contribution of 1,2,3,7,8,9-HxCDD. The patterns match to a high degree. A similar comparison was made for the milk sample from Côte d'Ivoire (Figure 4B). The pattern was highly comparable with the kaolinic clay causing the 2004 incident in the Netherlands and the clay sample collected from Côte d'Ivoire and labeled Kaolin. Also the pattern in the Mabele clay collected in the Netherlands and labeled Democratic Republic of the Congo showed a close resemblance to these clays.

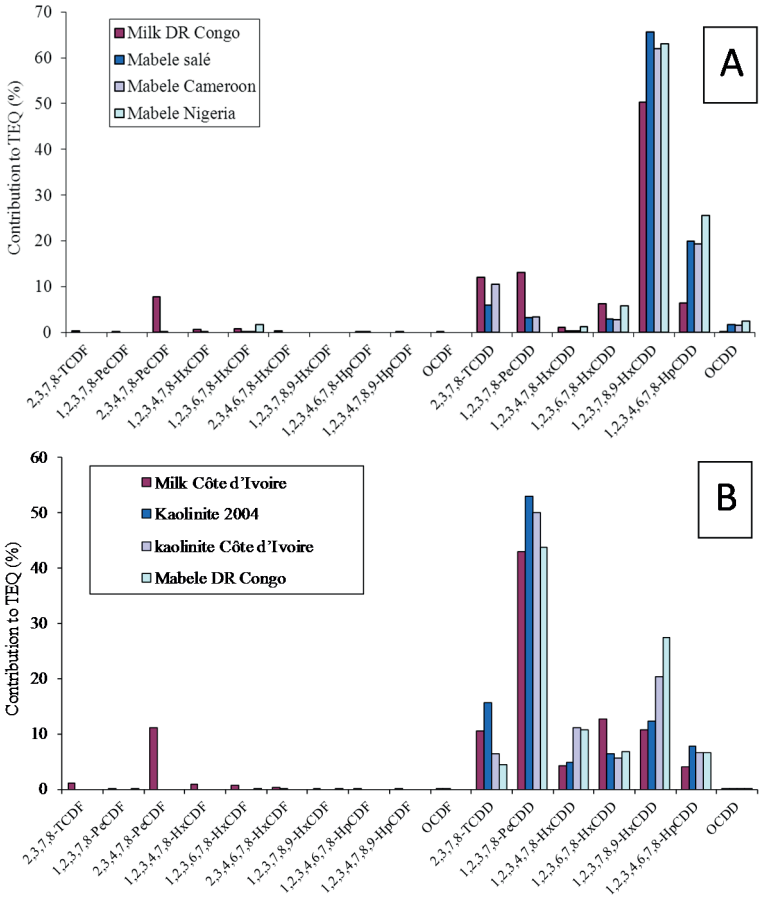

Figure 4. Comparison of the congener pattern in human milk from A, the Democratic Republic of the Congo with the patterns observed in three different Mabele clay samples and B, the congener pattern in human milk from Côte d'Ivoire with the patterns observed in two Kaolinite and one Mabele clay samples.

Discussion

High PCDD/F levels in pregnancy clays

Previous studies showed that clays used during pregnancy may contain high levels of heavy metals (Ibeanu et al. 1997; Dean et al. 2004; FSA 2007; Reeuwijk et al. 2013). The present study shows that some of these clays, termed as different types of Mabele and Kaolin, contain also high levels of PCDD/Fs (Table S-2). This was not unexpected considering previous observations of high levels of PCDD/Fs in Mississippi ball clay and kaolinic clay (Hayward et al. 1999; Ferrario et al. 2000; Jobst and Aldag 2000; Hoogenboom et al. 2010). Very typical for these contaminated clays is that they contain only PCDDs, although with different patterns. The kaolinic clay responsible for high levels of dioxins in bovine milk in the Netherlands in 2004, showed relative contributions of PeCDD, 1,2,3,7,8,9-HxCDD and TCDD to the TEQ-level of 53, 12 and 16% (Hoogenboom et al. 2010), that are also typical for other samples of this clay from the mine in the German Eifel. Four of the current clay products also showed a relatively high contribution of PeCDD and 1,2,3,7,8,9-HxCDD, but a relatively low contribution of TCDD. These included the samples from Côte d'Ivoire and Nigeria marketed as Kaolin. Two other samples, sold as Mabele in the Netherlands, showed a very high contribution of 1,2,3,7,8,9-HxCDD, followed by heptachlorodibenzo-p-dioxin (HpCDD). Another sample, obtained in Zimbabwe, is clearly marked by the high contribution of the hepta- and octa-CDD. However, due to the low TEF- values of these congeners, the TEQ-level in this sample was relatively low.

The highest level observed in the present study was 103 pg TEQ g^{-1} in a clay sample labeled Mabele salé, but based on previous studies, levels as high as 1 to 2 ng g^{-1} may also be encountered in ball clay and kaolinite (Ferrario et al. 2000; Hoogenboom et al. 2010). The PCDDs in these clays are thought to be formed under natural conditions in prehistoric ages, possibly by forest fires, volcanos or under high pressure and temperature in specific layers in the earth (Holmstrand et al. 2006; Gu et al. 2008; Horii et al. 2008). The source of the PCDDs detected in the contaminated pregnancy clays may be similar but further studies are required to reveal the origin of these clays and the contamination source.

Clays as a potential source of PCDD/Fs in human milk samples

PCDD/Fs accumulate in liver and especially fat tissues. However, it is well-known that milk is an important route for excretion of PCDD/Fs, both in lactating animals and in humans (Hoogenboom et al. 2010; Malisch et al. 2010). Human milk levels normally reflect the existing body burden, when expressed on a lipid base (body burden can be

defined as the concentration of PCDD/Fs in the body (JECFA 2002). Existing body burdens and hence mother milk levels reflect the low level exposure through the food. It was remarkable that the levels in the pooled milk of some African countries were much higher than those in other countries, suggesting a difference in the levels in the food. However, the close resemblance in the congener patterns in human milk samples with the highest PCDD/F levels, i.e., the samples from the Democratic Republic of the Congo and Côte d'Ivoire, with the patterns observed in certain clays (Figure 4) suggest a different possibility for the higher levels. Both samples showed the typical high contribution of PCDDs to the TEQ level. Furthermore, the pattern in the milk sample from Côte d'Ivoire was highly comparable with that of the kaolinic clay involved in the 2004 incident in the Netherlands, the pregnancy clay collected from Côte d'Ivoire (labeled as Kaolin) and the Mabele clay from the Democratic Republic of the Congo (Figure 4B). The human milk from the Democratic Republic of the Congo showed the very specific pattern with the high contribution of 1,2,3,7,8,9-HxCDD observed also in the Mabele clays labeled as salé and Cameroon, but collected in the Netherlands. Unfortunately, no clay was collected directly from the DR of the Congo. Overall, it cannot be excluded that the relatively high levels in these milk samples and the close resemblance in the patterns are caused by consumption of contaminated clays.

Potential risk of the use of contaminated clays during pregnancy

Exposure to TCDD has been shown to result in adverse effects on reproduction, development and endocrine functions (Van den Berg et al. 1998). Numerous animal studies with TCDD showed effects on the developing fetus, including a decreased sperm production of male rats exposed in utero (Faqi et al. 1998, Vandenberg et al. 2012). The exact mechanism behind these effects is still unclear but may be related to disturbances of the hormone status. Induction of certain biotransformation enzymes may cause the increased degradation of thyroid hormones. Also decreased testosterone levels have been observed in male rats. The studies on the decreased sperm production are the critical studies used by the Scientific Committee on Food (SCF) to derive the exposure limit (Tolerable Weekly Intake - TWI) of 14 pg TEQ kg bw^{-1} $week^{-1}$ (SCF 2001). In a similar manner, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) derived a Provisional Tolerable Monthly Intake (PTMI) of 70 pg TEF kg bw^{-1} $month^{-1}$ (JECFA 2002), for which additional reproductive toxicity studies were taken into account. These exposure limits were set for the chronic low-dose intake of PCDD/Fs and dl-PCBs through the daily intake of food contaminated with relatively low levels of these contaminants. They should prevent that women at child-bearing age have a body burden that exceeds critical levels as derived from the animal studies. This critical level is 4 ng TEQ kg bw^{-1}.

Regarding the above mentioned developmental effects, the question arises whether contaminated clay products used during pregnancy may have potential consequences for the developing child. Katulek et al. (2010) reported that in certain African regions between 46% and 73% of the pregnant and breastfeeding women regularly consume soil. Various other authors examined the consumption of clay during pregnancy and estimated the daily intake to be 30 to 80 g (Table S-1). At the highest observed level of 103 pg TEQ g^{-1} product, this would amount to an exposure of 3 to 8 ng TEQ per day, or 22 to 58 ng TEQ per week. A similar exposure would be obtained when using the 2005 TEFs instead of those from 1998 (see Results). Based on a body weight of 65 kg, this would be equivalent to 333 to 887 pg TEQ kg bw^{-1} $week^{-1}$, being 24 to 63 times higher than the TWI of 14 pg TEQ kg bw^{-1} $week^{-1}$. However, as the TWI was set for a chronic exposure, the question arises to what extent the intake of contaminated clay could affect the body levels of the mother and as such the exposure of the fetus. Based on an average background level for women in the Netherlands of 9 ng TEQ kg^{-1} fat, a body weight of 65 kg, 28% body fat and 75% of the PCDD/Fs and dl-PCBs in the fat (De Mul et al. 2008), the average total body burden anno 2008 was estimated at 220 ng TEQ. This amounts to 3.4 ng TEQ kg bw^{-1} (or 9 ng TEQ per kg fat), being just below the above mentioned critical limit. The intake of a single portion of the highest contaminated clay, containing 3-8 ng TEQ, would contribute only to a relatively small extent to the body burden of 220 ng TEQ. However, a prolonged daily intake during the first trimester (270-720 ng TEQ) or even the whole pregnancy (810-2160 ng TEQ) could lead to a serious elevation of the body burden (potentially up to 4-10x in the average Dutch woman). In African women, with apparently a much lower exposure through the food (based on the human milk levels), the relative impact on the body burden would be even much higher. The levels in the pooled milk samples from DR of the Congo and Côte d'Ivoire are comparable to the current levels observed in the Dutch women. However, it seems likely that some of the 50 milk samples in the pool contained much higher levels, especially when caused by the intake of contaminated clay. An increased body burden means an elevated exposure of the fetus but would also result in clearly elevated levels in the breast milk, thus prolonging the exposure of the child after birth. Of course this is based on a worst case scenario, although levels in certain clays might even be much higher than the ones observed in this study.

However, even when considering a relatively short exposure, it is important to realize that under normal conditions the fraction of the PCDD/Fs in the body that is actually circulating and causing the exposure of the fetus, is rather low. Levels of PCDD/Fs in the blood lipids are normally in equilibrium with those in the adipose tissue. Since blood contains only a small amount of fat (in total around 25 g), the total amount of PCDD/Fs in the blood at an average level of 9 pg TEQ g^{-1} fat observed in Dutch women would amount to 0.23 ng TEQ. The question is whether an intake of 3-8 ng TEQ

through a portion of highly contaminated clay could cause a serious increase of the blood levels and as such the increased exposure of the fetus. When deriving the TWI for chronic low-dose exposure, the original data were actually corrected by SCF by a factor of 2.3 since it was shown that the single high dose of TCDD applied in the animal studies resulted in relatively higher levels in the fetus than a similar dosage provided over a longer period (SCF 2001). It is also important to note that effects of TCDD on the fetus were in most animal studies observed after a single treatment of the mother during a specific period of the pregnancy (around gestational day 15). This indicates a short time period as the critical exposure window for the induction of reproductive toxicity. As such, comparing the contribution of the absolute amount of dioxins from the intake of a highly contaminated product solely with the existing and the critical body burden (4 ng TEQ kg bw^{-1}) may not take into account the temporary increase in the blood levels and as a result the increased exposure of the fetus during this critical window of development. Further studies are required to better understand this issue, including improvement and validation of existing physiologically based pharmacokinetic (PBPK) models with respect to the exposure of the fetus (RIVM-RIKILT 2009). Overall it seems clear that the intake of highly contaminated clays, especially during pregnancy, presents a clear risk for the unborn child. It should also be pointed out that in addition to PCDD/Fs, some of these clays were shown to contain high levels of metals and metalloids such as lead and arsenic (Reeuwijk et al. 2013). The combined effects of these compounds and PCDD/Fs are another matter of concern.

Application of a bioassay for screening of clay products

The present study shows that a bioassay such as the DR CALUX®-assay is a very useful tool to screen clay products for potentially high levels of PCDD/Fs. The test was capable of selecting the higher contaminated samples as confirmed by GC/HRMS. A possible exception seems sample S/N17 which showed a similar response as sample S/N13 (Figure 1), but a 15-fold lower GC/HRMS determined level (Table S-2). This can be explained by the high content of OCDD and HpCDD in this sample, which show relatively high relative potencies in the DR CALUX®-assay as compared to the assigned TEF value. The fact that three samples classified as slightly or non-suspected (Mabele Nigeria, S/N9 and S/N16) were confirmed by GC/HRMS to indeed have low TEQ levels, further demonstrates the suitability of the bioassay for screening purposes. The use of a relatively cheap bioassay for screening purposes could be relevant especially in developing countries. Confirmation of suspected samples by GC/HRMS is however prudent.

Conclusions

Consumption of several of the examined clays results in intakes above the TWI and could be a cause of concern for the developing fetus. The use of these contaminated clays, especially during pregnancy and lactation, should be discouraged. Although the benefits of breast feeding clearly outweigh the risks of these contaminants, a further reduction of contaminant levels in human milk is clearly a target for the current policies on reducing the levels of dioxins and related compounds in the food chain. Therefore, strict control on contaminated clay products should be carried out to prevent introduction, spreading and use of such products.

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CHAPTER 4

Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supplements sampled on the Dutch market

Based on:
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Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supplements sampled on the Dutch market

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Abstract

Herbal food supplements, claiming to enhance sexual potency, may contain deliberately added active pharmacological ingredients (APIs) that can be used for the treatment of erectile dysfunction (ED). The aim of this study was to determine whether herbal food supplements on the Dutch market indeed contain APIs that inhibit phosphodiesterase type 5 (PDE-5), such as sildenafil and analogous PDE-5 inhibitors. Herbal food supplements intended to enhance sexual potency (n=71), and two soft drinks, were sampled from 2003 up to and including 2012. In 23 herbal supplements nine different PDE-5 inhibitors were identified, in a few cases (n=3) more than one. The presence of these APIs was however not stated on the label. The concentrations of PDE-5 inhibitors per dose unit were analyzed. Furthermore, the potential pharmacological active properties of the detected PDE-5 inhibitors were estimated using data from scientific and patent literature regarding i) *in vitro* PDE-5 activity, ii) reported effective doses of registered drugs with PDE-5 inhibitor activity, and iii) similarity to other structural analogues. It was concluded that 18 of the 23 herbal food supplements, when used as recommended, would have significant pharmacological effects due to added APIs. Adequate use of existing regulation and control measures seems necessary in order to protect consumers against adverse effects of these products.

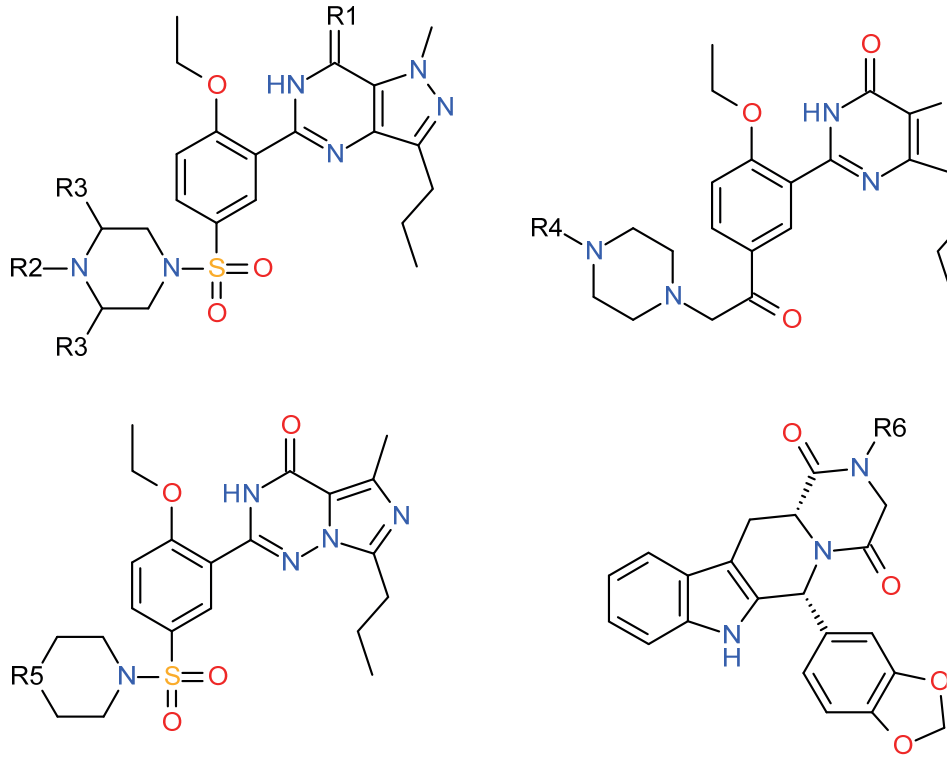
Introduction

Herbal food supplements that claim to enhance sexual potency are marketed worldwide and are readily available from Internet shops (FDA 2013, Lin et al. 2006; Singh et al. 2009; Venhuis and de Kaste 2012). These products generally claim to be 'all natural' but there are regular reports of adulterations with drugs for the treatment of erectile dysfunction (ED) (Blok-Tip et al. 2004; Lin et al 2006; Oh et al. 2006; Zou et al. 2006; Venhuis et al. 2008[a]; 2008[b]; 2011; 2012; Reepmeyer and Woodruff 2007; Reepmeyer et al. 2007; Hasegawa et al. 2008; Singh et al. 2009; Savaliya et al. 2010; Wollein et al. 2011; Kee et al. 2012; Lee et al. 2013). ED is defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance (NIH 1993).

In recent decennia synthetic drugs for the treatment of ED were developed, which started with the introduction of Viagra® in 1998 containing sildenafil (Figure 1) as the active pharmacological ingredient (API). This was followed in 2002 by Cialis®, containing the API tadalafil (Figure 1) and in 2003 by Levitra®, containing the API vardenafil (Figure 1). Sildenafil, tadalafil, and vardenafil, the APIs of these three registered drugs available on the Dutch market, belong to the class of nitric oxide–cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE-5) inhibitors (Singh et al. 2009; Venhuis and de Kaste 2012). In mammalian tissues, 11 PDE families have been identified with different tissue localizations (Corbin et al. 2002; Gresser and Gleiter 2002). PDEs vary in their substrate specificity, and PDE-5, PDE-6 and PDE-9 are known to be specific for hydrolysing cGMP (Corbin et al. 2002). PDE-5 is widely distributed in vascular and visceral smooth muscle cells, platelets, kidney, lung, and the lining of the blood vessels supplying the corpus cavernosum penis, the erectile tissue of the penis (Corbin et al. 2002; Pissarnitski 2006; Flores Toque et al. 2008). PDE-6 is located in the retina and has a role in visual transduction (Corbin et al. 2002). Sildenafil, vardenafil and tadalafil are moderately selective competitive inhibitors for PDE-5. Use of these APIs results in an increased smooth muscle relaxation, increased blood flow and an improved erection (Dale et al. 2000; Corbin et al. 2002; Pissarnitski 2006).

PDE-5 inhibitors have also vasodilatating effects on the pulmonary vessels and are therefore applied in registered drugs such as Revatio®, containing the API sildenafil, and Adcirca®, containing the API tadalafil, for the treatment of pulmonary hypertension. Because of the vasodilatating effects on the pulmonary vessels, PDE-5 inhibitors are contra-indicated in patients who are treated with other types of antihypertensives such as nitrates (eg. nitroglycerine, doxazosin and terazosin) (Boden et al. 2012; EMA 2013[a]). PDE-5 inhibitors are also contra-indicated in patients suffering from hypotension, but who are not treated for this (Kloner 2007). Reported side effects for the three market-

ed PDE-5 inhibitors include headache, facial flushing, nasal congestion, and dyspepsia (Gresser and Gleiter 2002).



sildenafil	R1=O, R2=CH_3, R3=H
homohydroxysildenafil	R1=O, R2=$HOCH_2CH_2$, R3=H
thiosildenafil	R1=S, R2=CH_3, R3=H
aildenafil	R1=O, R2=H, R3= CH_3
thioaildenafil	R1=S, R2=H, R3= CH_3
acetildenafil	R4=Et
noracetildenafil	R4=H
vardenafil	R5=EtN
piperidino-vardenafil	R5=CH_2
tadalafil	R6=CH_3
aminotadalafil	R6=NH_2

Figure 1. Chemical structures of sildenafil and analogous PDE-5 inhibitors

In 2002, shortly after the introduction of Viagra®, Cialis® and Levitra® on the Dutch market, the Netherlands Food and Consumer Product Safety Authority (NVWA) was alerted to the presence on the Dutch market of herbal food supplements claiming to enhance sexual performance, which contained undeclared sildenafil and also tadalafil.

At a later stage, also structural analogues of these three registered PDE-5 inhibitors were identified in herbal supplements on the Dutch market (Blok-Tip et al. 2004; Venhuis et al. 2008[a]; 2008[b]; 2011; Venhuis and de Kaste 2012). The use of non-registered ED products is growing worldwide. In literature at least 46 different analogues of sildenafil, vardenafil and tadalafil were identified until 2011. Most of these analogous PDE-5 inhibitors were found in food supplements, in counterfeits of registered medicines or in unapproved medicines (RIVM 2010; Venhuis and de Kaste 2012). These analogues are often the by-products of the typical drug development process. An analogue can be defined as a drug substance that is structurally and functionally comparable to a registered drug substance (RIVM 2010; Venhuis and de Kaste 2012).

As discussed above, health risks linked to the use of PDE-5 inhibitors may also arise from other side effects. For instance, aminotadalafil contains a reactive hydrazine structural element, which is suspected to have genotoxic potential (Orme et al. 2001; RIVM 2007; Häberli et al. 2010; Elder et al. 2011; Venhuis and de Kaste 2012). Moreover, acetildenafil is a non-selective PDE-5 and PDE-6 inhibitor. Inhibition of the latter is associated with temporary visual disturbances (RIVM 2007; Venhuis et al. 2008[a]).

Although herbal food supplements are covered by food law, medicinal products legislation takes precedence when the product can be classified as a medicinal product. When this is the case, only medicinal products legislation applies and any legal measure has to be taken on the basis of this legislation. From case law of the European Court of Justice it can be concluded that a product which is able to appreciably modify physiological functions by exerting a pharmacological action, taking into account its composition, including its content in active substances, and its intended use, could be classified as a medicinal product (European Court of Justice 2009). The aim of this study was to determine whether the observed API levels in various herbal food supplements on the Dutch market, such as sildenafil and analogous PDE-5 inhibitors, may result in significant pharmacological effects. Information on *in vitro* inhibitory effects on PDE-5, prescribed dose levels, and structural similarity in case of lack of information on activity, was used to assess the pharmacological effects. In addition, the existing regulatory framework was discussed.

Material and methods

Sampling

For the present study data from 71 herbal supplements and two soft drinks for enhancement of sexual potency, sampled by NVWA-inspectors from 2003 up to and including 2012, were evaluated. Sampling locations were identified by an Internet search

or selected from the NVWA inspection database. The sampling strategy was risk based. In the course of the survey inspectors became more experienced in finding these particular herbal supplements on the Dutch market. Key indicators for sampling were labeling and pricing. Products with two or more of the following characteristics were sampled with priority: i) a limited number of doses per container in combination with a relatively high price, statements on the labeling regarding ii) recommendations to use the product 1 or 2 hours prior to the intended effect (against ED), iii) contra-indications and adverse effects, and iv) the use of herbal ingredients from the Far East. The supplements consisted mainly of capsules, tablets, and liquids. All samples of herbal supplements were in pre-packaged form, and contained instructions for use. Brand names were anonymised by replacement with Roman numerals followed by the year of analysis.

Chromatographic screening for PDE-5 inhibitors

A HPLC-DAD-MS/MS method was developed to screen for the presence of PDE-5 inhibitors in herbal supplements (Venhuis et al. 2008[a]; 2008[b]; 2010; 2011). Briefly, half a dose unit was sonicated with methanol and then diluted 100 times using MeOH / 0,1% formic acid (buffered at pH = 4 with concentrated ammonia). After filtration the solution was analyzed by LC-MS/MS. Between each sample a blank was injected to asses carry-over. Compounds of interest were found by searching for marker fragments in MS/MS (Singh et al. 2009). Quantification was performed based on the Ultraviolet response using three or five point calibration curves. The Limit of Detection (LOD) of the compound for which the method was least sensitive (tadalafil) was < 0.1 mg per dose unit.

Assessment of the pharmacological effects of the APIs identified in the herbal supplements

In order to investigate whether medicinal products legislation applied to the sampled herbal supplements, the NVWA it was assessed in the present study whether their use would produce a significant pharmacological effect caused by the adulterant. Therefore, the pharmacological potencies and doses of the APIs identified were assessed. The pharmacological potency of the APIs found in the samples was estimated using different approaches. First by comparing the concentration of the API to data on the *in vitro* pharmacological activity of sildenafil and analogous PDE-5 inhibitors found in literature. Analogue concentrations that produce 50% inhibition of PDE-5 activity (IC_{50}) were compared by order of magnitude (pIC_{50}) (RIVM 2007; Venhuis et al. 2008[a]; 2008[b]). Furthermore, by comparing the dose of the API to the dose information for registered drugs with PDE-5 inhibitor activity available on the Dutch market, and other drugs with PDE-5

inhibitor activity identified from literature. Finally by assessing data on structural similarities between APIs from literature, including patent literature. For the estimation of the pharmacological effects of the analyzed APIs identified in the herbal supplements, we compared the combined data on the pharmacological potency to the dose level of the analyzed APIs resulting from intake of the herbal supplement.

Results and discussion

In total 71 herbal supplements and two soft drinks were analyzed for the presence of sildenafil and analogous PDE-5 inhibitors. Table 1 shows the APIs identified in the supplement and the dose levels, which were calculated from the analytically determined level. Table 2 shows the *in vitro* data identified in literature on the pharmacological activity of sildenafil and analogous PDE-5 inhibitors expressed as pIC_{50}, which is the negative logarithm of the IC_{50}. Dose information for registered PDE-5 inhibitors available on the Dutch market is shown in Table 3. In addition to sildenafil, eight different analogues of registered PDE-5 inhibitors were identified 27 times in total (Table 1). The structures of these APIs are also shown in Figure 1. The APIs were found in 23 herbal supplements with 12 different product names. In three herbal supplements (V, VI and XII) more than one API was detected. Sildenafil was found in only one herbal supplement (I) in 2003. Aildenafil (n=7) and acetildenafil (n=6) were the most frequently detected APIs in the 23 herbal supplements (Table 1). In addition, acetildenafil was found in two soft drinks, the labeling of which claimed that these drinks possessed libido enhancing effects (XIII and XIV).

Table 1. Dose levels of sildenafil and analogous PDE-5 inhibitors in herbal supplements sampled on the Dutch market.

Active Pharmaceutical Ingredient (API)	No. of herbal supplement with API	Dose levels (mg per dose unit)[a]
sildenafil	1	62.6 (I/2003)[a]
hydroxyhomosildenafil	4	33 (II/2004)[a], 87 (III/2007)[a], 65 (IV/2008)[a], traces (V/2008)[a,b,c]
thiosildenafil	1	0.2 (VI/2012)[a,d]
aildenafil	7	46 (VII/2008)[a], 49 (VIII/2008)[a], traces (V/2008[b], IX/2008, X/2008, XI/2008)[a,c], traces (XII/2010)[a,c]
thioaildenafil	1	82 (XII/2010)[a,e]
acetildenafil	8	4 (XIII/2004)[a,f], 7 (XIV/2004)[a,f], 60 (XV/2004)[a], 62 (XVI/2004)[a], 64 (XVII/2004)[a], 42 (XVIII/2005)[a], 52 (XIX/2006)[a], 36 (XX/2007)[a]
noracetildenafil	3	significant signals (XXI/2008, XXII/2008)[a,g], traces (VI/2012)[a,c,d]
aminotadalafil	2	0.3 (V/2008)[a,b], 53 (XXIII/2008)[a]
piperidino-vardenafil	2	2 (XXIV/2007)[a], 8 (XXV/2007)[a]

Notes:

[a] The names of the herbal supplements were replaced by Roman numerals, followed by the year of analysis of the herbal supplement

[b] Herbal supplement V/2008 contains traces of hydroxyhomosildenafil, traces of aildenafil and 0.3 mg per dose unit of aminotadalafil

[c] Trace levels were not quantified, but were below 0,1 mg

[d] Herbal supplement VI/2012 contains 0.2 mg per dose unit of thiosildenafil and traces of noracetildenafil

[e] Herbal supplement XII/2010 contains traces of aildenafil and 82 mg per dose unit of thioaildenafil

[f] Soft drinks

[g] Significant signals were found, but the levels were not assessed at the time of analysis

Assessment of the pharmacological effects of the APIs identified in the herbal supplements

Table 2 shows that the *in vitro* pharmacological activities of most PDE-5 inhibitors, expressed as pIC_{50}, are in the same order of magnitude. Only vardenafil is more potent by one order of magnitude. Therefore, the dose of the API is an important factor in determining whether the product would exert a significant pharmacological effect. Below it is discussed for each PDE-5 inhibitor identified in the current study, whether the herbal supplements which contained this API could result in a significant pharmacological effect.

Table 2. *In vitro* pharmacological activities of sildenafil and analogous PDE-5 inhibitors expressed as the concentration that produces 50% inhibition of PDE-5 catalytic potency (IC_{50}) and the negative logarithm of the IC_{50} (pIC_{50}).

Active pharmaceutical ingredient	IC_{50} (nM)	pIC_{50} (nM)	Relative pIC_{50} potency to sildenafil (*in vitro*)
sildenafil	7.1[a]	8.1	1.0
hydroxyhomosildenafil	3.4[a]	8.5	1.1
acetildenafil	7.6[a]	8.1	1.0
tadalafil	5.0[b]	8.3	1.0
vardenafil	0.7[c]	10.2	1.3

Notes:
[a] RIVM (2007)
[b] Patent: Pfizer-Research-and-Development-Company (1992)
[c] Dale et al. (2000)

Sildenafil was found in one herbal supplement (I) at a level of 62.6 mg per dose unit. Table 3 shows that for sildenafil the commercially available dose of a registered drug for the treatment of pulmonary hypertension is 20 mg (Revatio®) and the lowest available dose of sildenafil with a registered drug for the treatment of ED is 25 mg (Viagra®), which is lower than the estimated dose level of supplement I. It was therefore concluded that use of this herbal supplement can produce a significant pharmacological effect.

Hydroxyhomosildenafil was found in three herbal supplements (II, III and IV) at levels of 33, 87 and 65 mg per dose unit, respectively. Like sildenafil, hydroxyhomosildenafil is part of a patent (Pfizer 1994) in which these compounds are both claimed as ED drugs. Table 2 shows that hydroxyhomosildenafil, *in vitro*, is slightly more potent than sildenafil. A prodrug of hydroxyhomosildenafil, termed lodendafil carbonate, is commercially available in 80 mg tablets as an ED-medicine named 'Helluva' in Brazil (Flores Toque et al. 2008; Toque et al. 2008; Codevilla et al. 2011). All three doses found in the samples exceeded the lowest commercially available sildenafil doses of a registered drug (Table 3). It was concluded that the three herbal supplements investigated were likely to produce significant pharmacological effects.

Table 3. Registered drugs with PDE-5 inhibitors on the Dutch market and their indication, active pharmaceutical ingredient (API), available doses, and instruction for use.

Drug name	Manufacturer	Indication	API	Available doses (mg)	Instruction for use
Viagra®[a]	Pfizer	ED[f]	sildenafil	25, 50, and 100	As needed
Revatio®[b]	Pfizer	PH[g]	sildenafil	20	Chronic treatment, 3x 20 mg daily
Levitra®[c]	Bayer	ED[f]	vardenafil	5, 10, and 20	As needed
Cialis®[d]	Lilly	ED[f]	tadalafil	5, 10, and 20	As needed
Adcirca®[e]	Lilly	PH[g]	tadalafil	20	Chronic treatment, 2 x 20 mg daily

Notes:
[a] EMA (2013[a])
[b] EMA (2013[b])
[c] EMA (2013[c])
[d] EMA (2013[d])
[e] EMA (2013[e])
[f] Erectile Dysfunction
[g] Pulmonary Hypertension

Thiosildenafil was found in one herbal supplement (VI) at a level of 0.2 mg per dose unit. This API is reported to be more potent in both PDE-5 and PDE-6 inhibition than sildenafil (Cho et al. 2003). As the estimated dose of this API with this herbal supplement was much lower than the lowest commercially available sildenafil dose of a registered drug (Table 3), this supplement was not considered to induce significant pharmacological effects.

Aildenafil was found to be present in two herbal supplements (VII and VIII) at levels of 49 and 46 mg per dose unit, respectively. Patent and scientific literature showed that the pharmacological effects and potency of aildenafil were similar to sildenafil (Liu 2005; He et al. 2006; Li et al. 2007; Wang et al. 2007). As the dose levels found for aildenafil with these herbal supplements exceeded the lowest commercially available sildenafil dose of a registered drug (Table 3), these supplements were considered to produce significant pharmacological effects.

Thioaildenafil was found in one herbal supplement (XII) at a level of 82 mg per dose unit. This PDE-5 inhibitor was included in a patent from Li et al. (2007) describing pyrazolopyrimidinethione derivatives, and this API is chemically synthesised from aildenafil. Animal studies showed that thioaildenafil was slightly more effective as an ED drug than sildenafil (Liu 2005). Because the dose levels found for thioaildenafil considerably exceeded the lowest commercially sildenafil dose of a registered drug (Table 3), this herbal supplement was considered to produce significant pharmacological effects.

Acetildenafil was found in six herbal supplements (XV to XX) at levels in a range of 36 up to 64 mg per dose unit. Like sildenafil, acetildenafil is part of a patent (Pfizer 1994) in which these compounds are both claimed to be ED drugs. Table 2 shows that

acetildenafil, *in vitro*, is almost equipotent to sildenafil. Because the estimated doses of this API with all six herbal supplements exceeded the lowest commercially available sildenafil dose of a registered drug (Table 3), these herbal supplements were considered to produce significant pharmacological effects. Two soft drinks (XIII and XIV) contained 4 and 7 mg acetildenafil per can, respectively, which is below the lowest commercially available sildenafil dose of a registered drug (Table 3). As a result from this pharmacological effects were not expected from the consumption of one can of these soft drinks.

Noracetildenafil was found in three herbal supplements (VI, XXI and XXII). Trace levels (< 0,1 mg) were identified in supplement VI. Significant signals of noracetildenafil were found in the chromatogram of the supplements XXI and XXII, but the levels were not assessed at the time. Noracetildenafil is described in a patent (Pfizer 1994), which also includes sildenafil and acetildenafil. Significant pharmacological effects resulting from use of these herbal supplements were only expected for samples XXI and XXII.

Aminotadalafil was found to be present in two herbal supplements (V and XXIII) at levels of 0.3 and 53 mg per dose unit, respectively. Aminotadalafil is an analogue of tadalafil. This API is described in a patent in which it is reported to have a similar potency as tadalafil (Orme et al. 2001). Because the dose level of aminotadalafil estimated for herbal supplement XXIII (53 mg per dose unit) exceeded the highest commercially available dose of tadalafil of a registered drug (20 mg, Table 3), this herbal supplement was considered to produce significant pharmacological effects. For the herbal supplement (V) with a level of aminotadalafil of 0.3 mg per dose unit, no pharmacological effects were expected because the dose level found was far below the lowest commercially available tadalafil dose of a registered drug (5 mg, Table 3).

Piperidino-vardenafil was found in two supplements (XXIV and XXV), at levels of 2 and 8 mg per dose unit, respectively. In a patent on phenylsubstituted imidazotriazinones as phosphodiesterase inhibitors (Bayer 1999), piperidino-vardenafil is also described. Figure 1 shows that vardenafil and piperidino-vardenafil have very closely related molecular structures. We assumed piperidino-vardenafil and vardenafil to have similar pharmacological potencies. Based on the finding that the piperidino-vardenafil doses estimated for these supplements were close to the lowest commercially available vardenafil dose of a registered drug (5 mg, Table 3), we concluded that significant pharmacological effects resulting from the use of these two herbal supplements cannot be excluded.

From the assessment of the pharmacological effects of the APIs identified in the herbal supplements it was concluded that use of 18 herbal supplements, in which APIs were identified, could result in significant pharmacological effects.

It is of importance to note that other registered sildenafil analogues exist; including for example mirodenafil and udenafil (Göker et al. 2012), but their presence was not

detected in the herbal food supplements analyzed in the present study. In line with what others reported (Li et al. 2009; Göker et al. 2012) we occasionally detected dapoxetine, known to prevent premature ejaculation (McMahon 2012), but since it does not belong to the group of sildenafil analogues this was considered outside the scope of the present study.

Because our sampling protocol was specifically targeted at finding herbal supplements for libido enhancement that were more likely to be adulterated with PDE-5 than other herbal supplements for libido enhancement, these findings can not be extrapolated to all herbal supplements on the market that are claimed to enhance libido.

The labeling of some herbal supplements sampled in our study containing thiosildenafil, aildenafil, noracetildenafil and aminotadalafil suggested that these products were intended for women. However, the use of PDE-5 inhibitors on female libido is not supported by literature. In six herbal supplements (V, VI, IX, C, XI, XII) trace levels of hydroxyhomosildenafil, aildenafil and noracetildenafil were found. As trace levels are expected to have no pharmacological effects, the presence of these trace levels of PDE-inhibitors could indicate that the production process was not sufficiently controlled which might be an indication for non-GMP production (Good Manufacturing Practice), which is another reason for concern for these types of products.

Our finding that acetildenafil was present in two soft drinks in 2004, is noteworthy as well. Only one similar case was reported in Asia by Shin et al. (2004). These findings suggest that official control authorities should not rule out these food products when investigating products for adulteration with PDE-5 inhibitors.

A limitation of our study is that pIC_{50} values were not available for all analogues identified in the herbal supplements. Furthermore, comparing *in vitro* data may have limited predictive value as to the pharmacological effects *in vivo* (Corbin and Francis 2002; RIVM 2010; Venhuis and de Kaste 2012). For example, piperidino-vardenafil is reported to be more liphophilic than vardenafil (Pissarnitski 2006; RIVM 2007). Therefore, relative to vardenafil, piperidino-vardenafil may be absorbed better, thereby producing a significant pharmacological effect at a lower dose than would be derived by pIC_{50} comparison.

We also used data on the commercially available dose of registered drugs (Table 3) for the assessment of the pharmacological effect relevance of the levels of the APIs found in herbal supplements. Consequently, the low and trace levels of APIs found in herbal supplements V, VI, IX, X, XI, XII, XIII, XIV are not expected to be pharmacologically relevant. Their presence may be the result of a contamination. Manufacturers might not adequately clean their equipment between production runs. When produced under non-GMP (Good Manufacturing Practice) conditions, such low and trace levels may also be the result of inhomogeneous mixing of the API and the herbal matrix resulting in a

product composition that is variable. Non-conformity with GMP is another important concern for these types of products.

It is possible that use of herbal supplements containing APIs at lower dose levels than registered drugs containing this API would also result in pharmacological effects. However, reliable data from literature on this topic is lacking.

The current enforcement actions on adulterated herbal supplements take place when these products are already on the market. Therefore, consumers might already be at risk of the adverse effects of the supplements containing these APIs. It would be desirable that control authorities could prevent these products from entering the market. Alternatives to enforcement on the basis of medicinal products legislation, which requires investigation of the pharmacological action of the herbal supplements, might offer advantages over this method. Some alternative methods are employed in other countries. For example, imposing a generic ban on PDE-5 inhibitors in foods including herbal food supplements might simplify enforcement. A recent example of a related measure is the introduction of a generic ban on synthetic psychotropics in Austria covering all known classes of related psychoactive structures (Mayrhofer et al. 2012). Although such a measure would not prevent the entering on the market of adulterated herbal supplements, it could result in more efficient market control.

Another possible way forward to increase the safety of herbal supplements on the market may be the introduction of a more formal pre-marketing assessment for these products, which is currently in place in Singapore in order to regulate Chinese Proprietary Medicines. This type of medicines contains ingredients derived from (a combination) of plants, animals or minerals (Yee et al. 2005). A pre-marketing assessment system was advocated as well by the European Food Safety Authority (EFSA) (EFSA 2009).

To conclude, from the 71 herbal supplements sampled on the Dutch market, 23 supplements contained sildenafil and analogous PDE-5 inhibitors, from which most of the APIs found in our study are not registered as approved drugs. Enforcement actions based on medicinal products legislation were undertaken to remove 18 supplements from the market which contained sildenafil and analogous PDE-5 inhibitors, the use of which would result in significant pharmacological effects. Enforcement actions to remove such herbal supplements from the market on the basis of medicinal products legislation, require official control authorities to conduct investigations which can put a stress on their resources. In order to protect users of these herbal supplements the adequate use of the existing regulation and control measures seems necessary and the use of alternative methods for enforcement of adulterated herbal supplements should be explored.

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CHAPTER 5

Active pharmaceutical ingredients detected in herbal food supplements for weight loss sampled on the Dutch market

Based on:
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Active pharmaceutical ingredients detected in herbal food supplements for weight loss sampled on the Dutch market

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Abstract

Herbal food supplements, claiming to reduce weight, may contain active pharmacological ingredients (APIs) that can be used for the treatment of overweight and obesity. The aim of this study was to determine whether herbal food supplements for weight loss on the Dutch market contain APIs with weight loss properties. Herbal food supplements intended for weight loss (n=50), were sampled from August 2004 up to and including May 2013. A HPLC-DAD-MS/MS method was used to screen for the presence of the APIs in herbal supplements. In 24 samples the APIs sibutramine, desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant, sildenafil, and/or the laxative phenolphthalein were identified 41 times. The presence of these APIs was however not stated on the label. The potential pharmacological effects of the detected APIs were estimated using data from reported effective doses of approved drugs. Use of 20 of the 24 herbal food supplements, may result in potential pharmacological effects. Furthermore, risk assessment of phenolphthalein, a suspected carcinogen and found to be present in ten supplements, based on the Margin of Exposure (MOE) approach resulted in MOE values of 96-30,000. MOE values lower than 10,000 (96-220) were calculated for the daily intake levels of four out of these ten supplements in which phenolphthalein was found. However, taking into account that weight loss preparations may be used for only a few weeks or months rather than during lifetime, MOE values may be two to three orders of magnitude higher. The current study shows that the use of food supplements with sibutramine, DMS, DDMS, and/or phenolphthalein could result in pharmacological effects.

Introduction

Nowadays, overweight and obesity are a growing public health issue. The World Health Organization (WHO) estimates that more than 1.4 billion adults are overweight and at least 500 million people are obese. Overweight can be defined by a body mass index (BMI) between 25–30 kg m^{-2}, and obesity is defined by a BMI greater or equal to 30 kg m^{-2} (WHO 2013).

In the last decennia synthetic drugs for treatment of overweight and obesity were introduced on the market such as Reductil®, Meridia®, Reduxade®, and Zelium®, containing sibutramine as the active pharmacological ingredient (API), Acomplia®, containing rimonabant, Fentrate Retard® and Ponderal®, containing fenfluramine, Isomeride®, containing dexfenfluramine, and Xenical®, containing orlistat (Ioannides-Demos et al. 2006; RIVM 2009; EMA 2010[a]; EC 2013).

Herbal food supplements that claim to induce weight loss are marketed worldwide and are readily available from Internet shops (Jordan and Haywood 2007; De Carvalho et al 2011; Ozdemir et al. 2012; Ancuceanu et al. 2013). These products generally claim to be 'all natural' but there are frequent reports of adulterations with drugs for the treatment of overweight, obesity and constipation such as sibutramine, fenfluramine, rimonabant, orlistat, and phenolphthalein (Yuen et al. 2007; Zou et al. 2007; Wang et al. 2008; Chen et al. 2009; RIVM 2009; Tang et al. 2010; Vaysse et al. 2010; De Carvalho et al. 2012; Dunn et al. 2012; Stypulkowska et al. 2011; Phattanawasin et al. 2012; Ancuceanu et al. 2013).

The API sibutramine (Figure 1a), which was initially developed as an antidepressant, is a combined serotonin (5-HT) and noradrenaline (NA) re-uptake inhibitor. In the body sibutramine is rapidly metabolized through demethylation into desmethylsibutramine (DMS; Figure 1b) and didesmethylsibutramine (DDMS; figure 1c) (Nisoli and Carruba 2000; Kang et al. 2010[a]). DMS and DDMS are both pharmacologically active, inducing satiety and stimulation of thermogenesis (Glick et al. 2000; Nisoli and Carruba 2000; Ding et al. 2003; Padwal and Majumdar 2007). In 2010 the European Medicines Agency (EMA) recommended the suspension of the market authorizations for sibutramine in the European Union because of an increased risk of serious, non-fatal cardiovascular events, such as stroke or heart attack (EMA 2010[a]). Reported side effects of sibutramine include cardiovascular effects such as tachycardia and (arterial) hypertension (Ioannides-Demos et al. 2006; Padwal and Majumdar 2007; Müller et al. 2009; Cohen and Ernst 2010). Furthermore, several psychiatric symptoms are reported such as psychosis and (hypo)mania, occurring at dose levels of sibutramine in an estimated range of 2.8-60 mg (Taflinski and Chojnacka 2000; Litvan and Alcoverro-Fortuny 2007; Lee et al. 2008; Müller et al. 2009; Chen et al. 2010; Chong 2010; van Hunsel and van Grootheest 2011; Waszkiewicz et al. 2012).

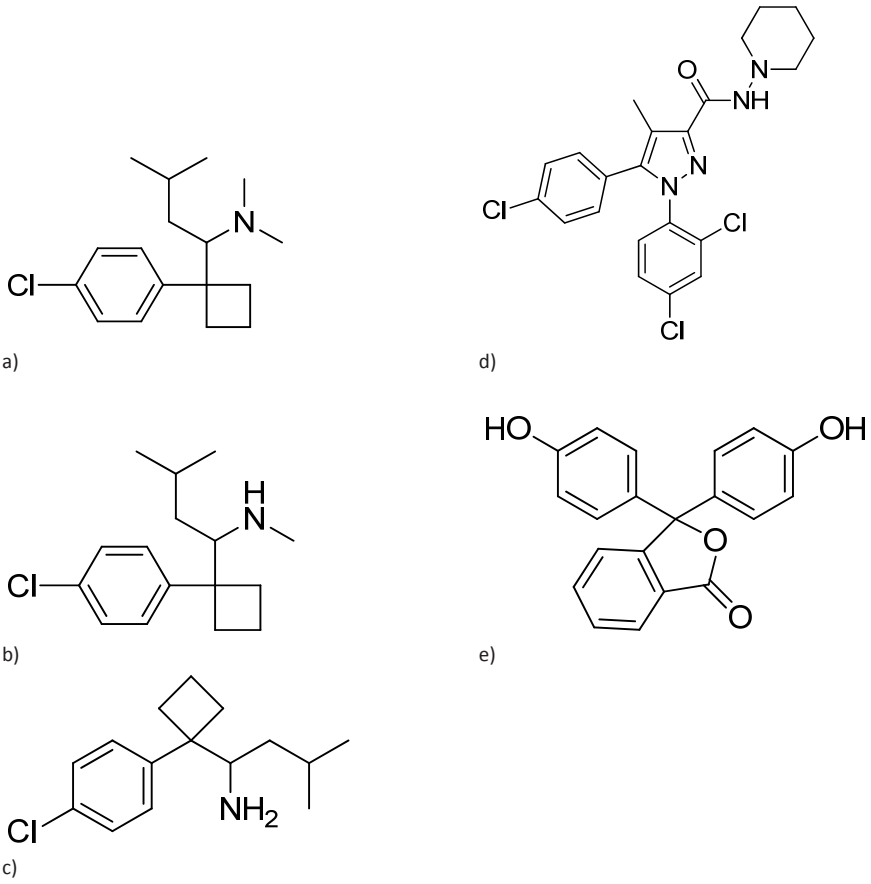

Figure 1. Chemical structures of **a)** sibutramine **b)** desmethylsibutramine **c)** didesmethylsibutramine **d)** rimonabant, and **e)** phenolphthalein.

Also adverse effects of DMS and DDMS have been clearly documented. Three cases of psychotic symptoms were linked to the use of slimming products containing DMS (Yuen et al. 2007; Chen et al. 2010), and cardiovascular effects were reported in a man who had used a herbal weight loss supplement containing DDMS (Fil et al. 2011).

Another weight loss drug, Acomplia®, which contains the API rimonabant (Figure 1d), was withdrawn from the European market at the end of 2008 because of severe side effects such as depression, and suicidal behaviour, and lack of efficacy (EMA 2007; EMA 2009; Venhuis et al. 2011; EC 2013). Rimonabant is a selective cannabinoid-1 receptor (CB1) blocker, which regulates food intake (Van Gaal et al. 2005; Padwal and Majumdar 2007). After the withdrawal of Acomplia® in 2008 counterfeit Acomplia® and

imitation products, containing rimonabant polymorphs, were reported to be available on the Internet (Venhuis et al. 2011).

Phenolphthalein (Figure 1e), which is a benzofuran derivate, was used in a number of authorized medicinal products for treatment of constipation (IARC 1999; NTP 2011). In 1996, the US National Toxicology Program (NTP) published data on the genotoxity of phenolphthalein and its carcinogenicity in laboratory animal studies (NTP 1996). In 1997, the EMA concluded that the National Competent Authorities should take these NTP data into account in their considerations of any restriction of phenolphthalein containing medical products on the national markets (EMA 1997).

In the Netherlands, the presence of sibutramine and phenolphthalein in a vitamin supplement and in capsules was first reported by the National Customs Laboratory in 2004 (RIVM 2009). Subsequently the Netherlands Food and Consumer Product Safety Authority (NVWA) monitored the presence of APIs in various herbal food supplements for weight loss on the Dutch market. The current study describes the results of this survey, and also evaluates the possible pharmacological relevance of dose levels detected based on data from reported effective doses of approved drugs. Furthermore, in the present study the risk assessment of the suspected carcinogenic effects of phenolphthalein was performed using the Margin of Exposure (MOE) approach (EFSA 2005). To this end, the MOE was determined by calculating the benchmark dose lower confidence limit for 10% extra cancer risk ($BMDL_{10}$) based on tumor data from animal studies and comparing this value to the intakes that would result from daily use of the herbal supplements at the recommended dosing.

Material and methods

Sampling

For the present study 50 herbal supplements for weight loss were sampled on the Dutch market by NVWA inspectors from August 2004 up to and including May 2013. Sampling locations were identified by an Internet search or selected from the NVWA inspection database. The sampling strategy was partly risk based. Inspectors collected products based on experience and on the basis of reports and alerts of national and international health authorities concerning adulterated food supplements on the market and reports of side effects linked to specific products. These national and international reports and alerts included an official warning in the Netherlands of side effects of a food supplement containing green coffee (IGZ 2010) and consumer warnings from health authorities such as from Australia (Australian government 2007), Canada (Health Canada 2006), UK (MHRA 2010), and the US (FDA 2013). These reports and alerts

pointed to herbal supplements that might contain APIs and these were indicated for sampling in our sampling strategy. Furthermore, the sampling strategy was also based on reports of side effects which were directly reported by consumers to the NVWA and on reports of side effects which were received from the Netherlands Pharmacovigilance Centre (Lareb). Moreover, previous analyses by the Dutch Customs Laboratory of food supplements imported into the Netherlands and findings of the APIs in these products were also taken as a basis for the sampling strategy. Additionally, one of the indicators for identifying suspected herbal supplements were user reports on Dutch Internet forums in which users reported side effects resulting from the use of herbal slimming products.

The sampled supplements consisted mainly of capsules, tablets, and sachets with powder. All samples of herbal supplements were in pre-packaged form, and contained instructions for use. Brand names were anonymized by replacement with Roman numbers.

Chromatographic screening for APIs in the herbal supplements

An HPLC-DAD-MS/MS method was developed to screen for the presence of APIs in herbal supplements (Venhuis et al. 2011). Briefly, half a dose unit was sonicated with methanol and then diluted 100 times using MeOH / 0,1% formic acid (buffered at pH = 4 with 35% concentrated ammonia). After filtration over a 0.45 µm filter (Whatman GmbH, Dassel, DE) the solution was analyzed for common weight-loss drugs by LC-DAD-MS/MS. Between each sample a blank was injected to prevent carry-over. Compounds of interest were found by searching for MH^+ ions (full scan) and marker fragments (MS/MS) (Venhuis et al. 2011). Quantification was performed based on the UV response using three or five point calibration curves. Because the sample matrices were complex and highly variable, each negative screening result was challenged by spiking the sample with a pharmacologically irrelevant level of sibutramine. In most instances spiked sibutramine could be identified at a level of 0.1 mg $dose^{-1}$. In a few cases the matrix required spiking at a level of up to 0.6 mg $dose^{-1}$ before sibutramine could be identified.

Assessment of the pharmacological effects of the APIs identified in the herbal supplements

In order to investigate whether the use of an analyzed herbal supplement would be of pharmacological relevance, the pharmacological potencies and doses of the APIs identified in the herbal supplements were assessed. The pharmacological potency of the APIs found in the sample was estimated by comparing the daily intake of the API that would

result from its use according to the manufacturer's prescriptions to the dose information for registered drugs. Furthermore, the recommended daily dose of the herbal supplement was compared to the lowest commercially available daily dose of a drug containing the API. The recommended daily dose of the herbal supplement was identified from the label, or from an Internet search. For the calculation of the resulting intake of the API we selected the upper range of the recommended daily dose of the herbal supplement. For the herbal supplements for which no recommended daily dose could be identified we assumed the recommended daily dose to be one dosing unit day^{-1}. The pharmacological potencies of DMS and DDMS were estimated by comparing data from (patent) literature on pharmacological profiles of sibutramine to DMS and DDMS.

Risk assessment of phenolphthalein identified in the herbal supplements

For the risk assessment of phenolphthalein detected in the herbal supplements, we applied the Margin of Exposure (MOE) approach (EFSA 2005). Benchmark dose (BMD) and $BMDL_{10}$ values were obtained by fitting the carcinogenicity data obtained from literature to a number of different mathematical models using BMDS software, version EPA 2.4 (EPA 2013). The following models were used: Gamma, Logistic, LogLogistic, LogProbit, Multistage-cancer, Multistage, Probit, Quantal-Linear and the Weibull model. BMDS software was applied without model restrictions, using default setting of extra risk type, a 95% confidence level, and a Benchmark response (BMR) of 10%. The $BMD(L)_{10}$ values derived from the different fitted models were only accepted if the fit of the selected model was not of poorer quality than that of the so-called full model representing a perfect fit to the dose-response data. For this purpose, the p-value was taken into account with a value below 0.05 resulting in model rejection. For calculation of the MOE the lowest $BMDL_{10}$ value was selected (EFSA 2005). The estimated daily intake (EDI) for phenolphthalein from the herbal supplements was calculated based on the concentration of phenolphthalein detected in the supplement and the recommended daily dose indicated on the label, using a default body weight of 70 kg (EFSA 2012). The MOE values were calculated by dividing the $BMDL_{10}$ by the EDI (EFSA 2005).

Results

Presence of APIs in herbal supplements

In total 50 herbal supplements for weight loss were analyzed for the presence of APIs. Table 1 shows the APIs identified, the recommended daily dose in dose units day^{-1} for the respective herbal supplements and the dose levels of the APIs that would result

from this level of use. The latter values were calculated from the analytically determined level of the API and the recommended daily use of the herbal supplement indicated on the label or identified from an Internet search. For some herbal supplements no recommended daily dose could be identified, and for these herbal supplements the recommended daily dose was set to one dosing unit day^{-1}. Sibutramine, DMS, DDMS, phenolphthalein, sildenafil or rimonabant were identified in 24 herbal supplements with 12 different brand names. In 11 herbal supplements more than one API was detected. Sibutramine was found in 17 herbal supplements and was the most frequently detected API. In addition, phenolphthalein, DDMS, and DMS were found in ten, six, and four herbal supplements, respectively. Rimonabant was found in one herbal supplement. Additionally, in two herbal supplements (XVIII and XIX) trace levels of the PDE-5 inhibitor sildenafil were found, and in one herbal supplement (XVI) a dose level of 0.9 mg dosing $unit^{-1}$ of sildenafil was found (Table 1). The APIs fenfluramine and N-nitrosofenfluramine were not found.

Table 1. Dose levels of the Active Pharmaceutical Ingredients (APIs) sibutramine (sib), desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant (rim), sildenafil (sil) and phenolphthalein (phen) in herbal supplements sampled on the Dutch market.

Herbal supplement[a]	Brand name[b]	Year of sampling	Analytically determined concentration (mg dosing $unit^{-1}$)[c]	Recommended daily dose (dosing unit day^{-1})	Estimated daily dose level (mg day^{-1})[d]
I	A	2010	11 (sib)	1	11 (sib)
II	A	2010	21(sib)	1	21 (sib)
III	A	2010	21 (sib)	1	21 (sib)
IV	A	2010	19 (sib)	1	19 (sib)
V	B	2011	18 (sib)	1-2	18-36 (sib)
VI	B	2011	3.7 (DDMS); 0.3 (phen)	1-2	3.7-7.4 (DDMS); 0.3-0.6 (phen)
VII	B	2011	3.6 (DDMS); 0.2 (phen)	1-2	3.6-7.2 (DDMS); 0.2-0.4 (phen)
VIII	B	2011	3.7 (DDMS); 0.2 (phen)	1-2	3.7-7.4 (DDMS); 0.2-0.4 (phen)
IX	B	2011	8 (sib)	1-2	8-16 (sib)
X	C	2011	2 (sib)	2	4 (sib)
XI	C	2011	13 (sib)	2	26 (sib)
XII	D	2011	16.8 (sib)	1	16.8 (sib)
XIII	E	2011	6 (sib); 43 (phen)	1	6 (sib); 43 (phen)
XIV	F	2012	16 (DDMS)	0.5-1	8-16 (DDMS)
XV	F	2012	16 (DDMS)	0.5-1	8-16 (DDMS)

Herbal supplement[a]	Brand name[b]	Year of sampling	Analytically determined concentration (mg dosing unit^{-1})[c]	Recommended daily dose (dosing unit day^{-1})	Estimated daily dose level (mg day^{-1})[d]
XVI	G	2012	traces (DDMS); 43 (phen); 0.9 (sil)	1[e]	43 (phen); 0.9 (sil)
XVII	H	2012	12 (sib); 27 (phen)	1	12 (sib); 27 (phen)
XVIII	I	2012	10 (sib); traces (DMS); traces (sil)	1[e]	10 (sib)
XIX	J	2012	15 (sib); 31(phen); traces (sil)	1-2	15-30 (sib); 31-62 (phen)
XX	K	2012	20 (sib)	1[e]	20 (sib)
XXI	A	2012	0.3 (rim)	1[e]	0.3 (rim)
XXII	A	2013	traces (sib); 0.1 (DMS); traces (phen)	1[e]	0.1 (DMS)
XXIII	A	2013	traces (sib); 0.2 (DMS); traces (phen)	1[e]	0.2 (DMS)
XXIV	L	2013	traces (sib); 0.1 (DMS); traces (phen)	1[e]	0.1 (DMS)

Notes:

[a] The samples of the herbal supplements were numbered by Roman numbers

[b] The brand names of the herbal supplements were replaced by alphabetic letters

[c] Sibutramine, DMS, and DDMS are reported as a free base

[d] Analyzed dose level multiplied by recommended daily dose

[e] No recommended daily dose could be identified; recommended daily dose set on 1 dosing unit day^{-1}

Assessment of the pharmacological effects of the APIs identified in the herbal supplements

Below it is discussed for each API identified in the current study, whether the use of the herbal supplements which contained the APIs identified would be of pharmacological relevance.

Sibutramine

Sibutramine was found in 14 samples at levels that would result in estimated daily dose levels of 4 up to 36 mg sibutramine (as a free base) day^{-1}. Additionally, in three herbal supplements traces of sibutramine were found (Table 1). For treatment of weight loss sibutramine-HCl is commercially available as a registered drug in doses of 10 and 15 mg, and prescribed at one dosing unit day^{-1} (EMA 2002; EMA 2010[b]). For estimating the potential pharmacological effect of the herbal supplements we selected the lowest commercially available dose of 10 mg sibutramine-HCl, which corresponds to 8.3 mg of

the free base. The use of 12 herbal supplements (I-V, IX, XI, XII, XVII-XX) at the recommended daily dosing would exceed the lowest commercially available daily recommended sibutramine dose as the free base (8.3 mg) of this formerly registered drug, and these 12 herbal supplements were thus considered to induce pharmacological effects.

DMS

DMS was found to be present in three samples at levels that would result in estimated daily dose levels of 0.1 (XXII, XXIV) and 0.2 mg day^{-1} (XXIII) when used as recommended. Additionally, one herbal supplement contained traces of DMS (Table 1). From a literature search we concluded that DMS and DDMS have similar pharmacological effects as sibutramine *in vivo* (Jerussi et al. 2001; Kang et al. 2010[b]). Furthermore, based on the differences of sibutramine, DDMS and DMS on the uptake of norepinephrine, serotonin and dopamine, we estimated DMS and DDMS to be up to 50-fold more potent than sibutramine (Glick et al. 2000). Patent literature suggests an effective dose for DDMS as off 0.2 mg day^{-1} (Barberich 2005) in the treatment of sleeping disorders. Because of the similarities in the pharmacological profiles of DDMS and DMS we assume both APIs are pharmacological active at a dose ≥ 0.2 mg day^{-1}. Because the highest estimated daily dose of DMS was 0.2 mg day^{-1} (XXIII) we considered that use of this supplement is likely to induce pharmacological effects.

DDMS

DDMS was found to be present in five samples (VI-VIII, XIV, and XV) at levels that, when used as recommended, would result in an estimated intake of 3.6-16 mg day^{-1} (Table 1). Additionally, one herbal supplement (XVIII) contained traces of DDMS (Table 1). Because the lowest estimated daily dose of this API with herbal supplement VII (3.6 mg day^{-1}) is above the lowest dose of DDMS (0.2 mg day^{-1}) of which pharmacological effects are expected, the use of these five supplements as recommended at estimated daily DDMS levels from 3.6 to 16 mg day^{-1} was considered to induce pharmacological effects.

Rimonabant

Rimonabant was found in one herbal supplement (XXI) at a level that would result in an estimated daily dose level of 0.3 mg day^{-1} when used as recommended. The drug Acomplia® is commercially available in doses of 20 mg rimonabant per dosing unit, taken once daily (EMA 2013). Because the estimated daily dose level of rimonabant in herbal supplement XXI appears to be far below the commercially available rimonabant

dose of a registered drug amounting to 20 mg rimonabant day^{-1}, it can be concluded that use of this herbal supplement as recommended, is unlikely to result in pharmacological effects.

Sildenafil

Sildenafil, which is a phosphodiesterase type 5 inhibitor, was identified on the Dutch market in herbal food supplements used to enhance sexual potency (Reeuwijk et al. 2013). For sildenafil the commercially available dose of a registered drug for the treatment of pulmonary hypertension is 20 mg (Revatio®) and the lowest available dose of sildenafil with a registered drug for the treatment of erectile dysfunction is 25 mg (Viagra®) (Reeuwijk et al. 2013), which is higher than the analyzed dose levels of supplements XVI (0.9 mg day^{-1}), XVIII (traces), and XIX (traces) (Table 1). It was therefore concluded that use of these three herbal supplements as recommended, is not expected to produce a pharmacological effects.

Phenolphthalein

Phenolphthalein was found in seven herbal supplements at levels that would result in estimated daily dose levels ranging from 0.2 up to 62 mg day^{-1} (Table 1). EMA (1997) reported recommended clinical oral doses of phenolphthalein to be 50 to 200 mg, and an anticipated human intake in normal users of 4 mg phenolphthalein kg bw^{-1} day^{-1}, which is equivalent to 280 mg day^{-1} for a 70 kg adult. Furthermore, NTP (2011) reported oral dose levels of phenolphthalein available as over the counter drugs ranging from 30 to 200 mg for adults. Doses for children, aged from two to eleven years, were reported to be 15 to 60 mg (NTP 2011). Additionally, IARC (1999) reported phenolphthalein to be available as tablets in a range from 6.5 to 200 mg. For the assessment of the pharmacological effect of phenolphthalein present in the herbal supplements, we assumed the lowest daily dose level of phenolphthalein to be 6.5 mg phenolphthalein day^{-1}, which was reported by IARC (1999). Estimated daily dose levels of four herbal supplements (XIII, XVI, XVII, and XIX) exceeded this selected lowest reported available daily dose (6.5 mg day^{-1}), and it was therefore considered that use of these herbal supplements can produce a pharmacological effect.

Altogether, from the assessment of the pharmacological effects of the APIs identified in the herbal supplements it was concluded that use of 20 out of 24 herbal supplements, in which the APIs sibutramine, DMS, DDMS, and/or phenolphthalein were identified, could result in pharmacological effects.

Risk assessment of phenolphthalein identified in the herbal supplements

Aside from its pharmacological effects, phenolphthalein also possesses carcinogenic potency (NTP 1996; 2011; IARC 1999). For the risk assessment of phenolphthalein we used data from the study of Dunnick and Hailey (1996) and NTP (1996) on the incidence of histiocytic sarcomas in B6C3F1 male mice exposed in four dose groups to phenolphthalein for 104 weeks by feed (Table 2). Table 3 shows the results from the BMD analysis of the data from the study of Dunnick and Hailey (1996) and NTP (1996). $BMDL_{10}$ values were calculated and these were in a range of 85-557 mg kg bw^{-1} day^{-1}. For the calculation of the MOE values resulting from exposure to phenolphthalein in herbal supplements we used the lowest $BMDL_{10}$ value which was 85 mg kg bw^{-1} day^{-1}. The MOE values obtained using the estimated daily intakes (EDI) that would result from use of the phenolphthalein containing herbal supplements varied from 96 to 30,000. Use as recommended of four of the herbal supplements containing phenolphthalein (XIII, XVI, XVII, and XIX) would result in MOE values ranging from 96 to 220.

Table 2. Data on the incidence of hystiocytic sarcoma in $B6C3F_1$ male mice exposed to increasing dose of phenolphthalein for 104 weeks by feed (Dunnick and Hailey 1996; NTP 1996) used in the current study for the BMD analysis.

Dose (mg kg bw^{-1} day^{-1})	No. of animals	Incidence hystiocytic sarcoma
0	50	1
300	50	3
600	50	11
1200	49	12

It must be emphasized that in this approach MOEs are calculated assuming lifetime exposure while herbal food supplements for weight loss may only be used during relatively short periods for several weeks or months. For this reason, calculation of the MOEs assuming lifetime exposure at the estimated daily intakes might overestimate the potential risk for human health. However, a general framework for taking intermittent and/or short-term instead of lifetime exposures to compounds that are both genotoxic and carcinogenic into account in the safety assessment is currently not in place. Felter et al. (2011) recently proposed to use the principle of Haber's Rule for assessing the risk from less-than lifetime exposures to carcinogens, provided that chemical-specific carcinogenicity data are available and that data support a linear dose-response relationship (Felter et al. 2011). Haber's Rule assumes that the acceptable cumulative lifetime exposure can be averaged over the duration of short-term exposure, suggesting that higher daily intakes are acceptable when short-term exposure is considered

(Felter et al. 2011). Applying Haber's Rule to assess the potential risk for short-term exposure during a period of several weeks or months on an estimated life expectancy of 75 years may result in MOE values that are two to three orders of magnitude higher than those obtained when assuming lifetime (75 years) daily use of the herbal supplements.

Table 3. Results from a BMD analysis of the data on the incidence of hystiocytic sarcoma in $B6C3F_1$ male mice exposed to phenolphthalein (NTP 1996; Dunnick and Hailey 2006) using BMDS software version 2.4, a BMD of 10% extra risk and default settings.

Model	Restriction	No. of parameters	Log Likelihood	p-value	Accepted[a]	BMD_{10} (mg kg bw^{-1} day^{-1})	$BMDL_{10}$ (mg kg bw^{-1} day^{-1})
Null		1	-79.011	-	-	-	-
Full		4	-69.872	-	-	-	-
Gamma	none	3	-70.989	0.14	Yes	403	85
Logistic	na[b]	2	-72.501	0.07	Yes	690	557
LogLogistic	none	3	-70.935	0.15	Yes	401	98
LogProbit	none	3	-70.838	0.17	Yes	408	117
Multistage-cancer	na[b]	2	-70.993	0.33	Yes	415	292
Multistage	none	3	-70.902	0.15	Yes	357	185
Probit	na[b]	2	-72.227	0.10	Yes	648	519
Quantal-Linear	na[b]	2	-70.993	0.33	Yes	415	292
Weibull	none	3	-70.985	0.14	Yes	398	89

Notes:

[a] Fitted model not significantly different (worse) than the full model at $p<0.05$

[b] not applicable

Discussion

In this study 50 herbal supplements for weight loss available on the Dutch market were investigated for the presence of APIs. In 24 samples the APIs sibutramine, DMS, DDMS, rimonabant, sildenafil, and/or the laxative phenolphthalein were identified 41 times, and in 11 herbal supplements more than one API was identified. Possible pharmacological effects, and for phenolphthalein also toxicological effects, resulting from the presence of these APIs in the herbal supplements were evaluated. It was found that the use as recommended of 20 out of the 24 herbal supplements, in which sibutramine, DMS, DDMS, and phenolphthalein were identified, would result in estimated daily dose levels that could result in pharmacological effects. Furthermore, the use of four out of ten herbal supplements containing phenolphthalein would result in daily intake levels that

result in MOE values in a range of 96-220, being lower than 10,000. For short term-exposure (a few weeks or months) MOE values may be two or three orders of magnitude higher.

Estimation of pharmacological effective doses of the APIs found

To investigate whether medicinal products legislation could be applied to the sampled herbal supplements, we assessed whether their use would produce a pharmacological effect. For this, we compared the dose level of APIs resulting from the recommended use of the herbal supplements to the lowest dose level of commercially available drugs containing sibutramine, rimonabant, sildenafil, and phenolphthalein, identified in literature. We concluded that when the lowest dose level of the commercially available drugs was exceeded pharmacological effects could be expected. However, it cannot be excluded that dose levels resulting from the use of herbal supplements at estimated daily dose levels that are below the lowest commercially available daily dose will also produce pharmacological effects. Thus estimates presented for the possible pharmacological effects from the herbal supplements containing these APIs may be underestimates.

A limitation of our study in this respect is that for the APIs DMS and DDMS no commercially available doses exist or have existed. Furthermore, reliable data from literature on the pharmacologically active doses of DMS and DDMS are lacking. Relative to sibutramine, we estimated that DMS and DDMS could produce pharmacological effects at doses that are 50 fold lower (Glick et al. 2000). Based on this estimate we assumed the lowest pharmacologically effective dose of DMS and DDMS to be 50 fold lower than the lowest pharmacologically effective dose of sibutramine (8.3 mg day^{-1}), thus equating to 0.2 mg day^{-1}. It should be noted that 0.2 mg day^{-1} is an estimation of the pharmacological effective dose of DMS and DDMS, and this dose may be an underestimation or an overestimation.

Herbal supplements containing a combination of sibutramine, DMS, or DDMS with phenolphthalein

In our study the combination of sibutramine, DMS or DDMS with phenolphthalein was found in ten herbal supplements. The presence of the combination of sibutramine with phenolphthalein in a herbal supplement was reported earlier in the Netherlands (RIVM 2009). Also in other countries such as in the US (Dunn et al. 2012), France (Vaysse et al. 2010), China (Wang et al. 2008), and Hong Kong (Yuen al. 2007) combinations of sibutramine, DMS or DDMS with phenolphthalein were reported. Laxatives, like phenolphthalein are considered not very effective as weight loss drugs (Martin et al. 1998).

However, a reported side effect of sibutramine is constipation (Nisoli and Carruba 2000; Wilfley et al. 2008; Müller et al. 2009), and phenolphthalein might have been added because of its laxative properties.

Levels of sibutramine found in herbal supplements with an identical brand name

In our study we identified sibutramine levels in samples with a similar brand name which varied from trace levels to levels that would result in an estimated daily dose of 21 mg sibutramine in the herbal supplements I-IV, XXII, and XXIII (Table 1). Furthermore, in a sample of a herbal supplement with the same brand name (XXI), sibutramine could not be identified. Moreover, we reported that in two brands of adulterated dietary supplements used for weight loss, the dose units in one package contained either no API, one API, or different APIs such as sibutramine, DMS and DDMS, and at different dosages (Venhuis et al. 2013). These results indicate variability in the contamination of herbal supplements of a specific brand name suggesting the APIs are not present at a systematic or fixed level. This suggests that the risks associated with use of these herbal supplements will be variable because of differences in the levels and type of APIs present. In order to further quantify these differences, future work could focus on analysis of composite samples accompanied by analysis of several samples from individual dose units in one package and from different packages from a specific brand.

Trace levels of APIs

Trace levels of sibutramine, sildenafil, and phenolphthalein were found in some herbal supplements (XVIII, XIX, XXII, XXIII, XXIV), and trace levels of DMS were also found in one product (XVIII). These trace levels are not expected to be pharmacologically relevant. Furthermore, the presence of trace levels of APIs is most likely a quality problem associated with manufacturing deficiencies such as inadequate cleaning of the equipment between production runs, or inhomogeneous mixing of the API and the herbal matrix resulting in a variable product composition. It is of importance to note that trace levels of APIs, in combination with other APIs present at higher levels, might add to the pharmacological effect of the supplement. This possible additive pharmacological effect of combinations of different APIs remains to be investigated.

Sampling protocol

In our study we applied a targeted sampling protocol. Inspectors sampled products based on experience and on the basis of reports of national and international health authorities concerning adulterated food supplements on the market, and reports of

side effects linked to specific products. Prior to analysis we searched the Internet for more information on the sampled food supplements in order to assess the likelihood of APIs being present. In several cases we found in Internet forums on losing weight, reports of consumers on side effects of the sampled products which were later analytically shown to contain APIs. These consumer reports might be used as a crude indicator for the presence of APIs.

Risks phenolphthalein

For the risk assessment of phenolphthalein, which is expected to be a possible human carcinogen (IARC 2000), we calculated $BMDL_{10}$ values which were based on the incidence of hystiocytic sarcomas in B6C3F1 male mice reported in the study of Dunnick and Hailey (1996) and NTP (1996) (Table 3). Although we are aware that this study was based on a limited amount of dosing groups, we decided to use the study for calculation of the $BMDL_{10}$ because no other relevant studies on the carcinogenic properties of phenolphthalein were available. Whenever more precise toxicological data will become accessible, the calculated $BMDL_{10}$ value and resulting MOE values can be refined.

The MOE approach is recommended by the European Food Safety Authority (EFSA) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for priority setting by risk managers (EFSA 2005; JECFA 2005). EFSA (2005) considers that a MOE of 10,000 or higher, which is based on the $BMDL_{10}$ from an animal study, would be of low concern from a public health point of view and might be considered as a low priority for risk management actions. Use of four herbal supplements containing phenolphthalein (XIII, XVI, XVII, and XIX) at the recommended daily doses would result in MOE values lower than 10,000 (96-220). However, assessing the risk from less-than lifetime exposures and considering the use of the herbal supplements during a period of only a few weeks or months will result in MOE values which may be 2 to 3 orders of magnitude higher.

Side effects of API levels found in sampled herbal supplements

Results of the present study indicate that consumers of weight loss herbal supplements should be aware that these products can contain APIs. Because the current study was based on a targeted sampling protocol, the current results can not be extrapolated to the entire market of weight loss food supplements. However, when the product produces significant results, such as weight loss, users should also be attentive to side effects, which might be caused by APIs. For sibutramine present in herbal supplements side effects such as psychiatric symptoms are reported at dose levels in an estimated range of 2.8-60 mg (Taflinski and Chojnacka 2000; Litvan and Alcoverro-Funy 2007; Lee

et al. 2008; Müller et al. 2009; Chen et al. 2010; Chong 2010; van Hunsel and van Grootheest 2011; Waszkiewicz et al. 2012). In our study sibutramine was found in 14 samples at levels that would result in estimated daily dose levels of 4 up to 36 mg sibutramine day^{-1} when used according to recommendations. The lowest estimated daily dose level (4 mg day^{-1}) is above the lowest dose of this API at which side effects are reported (2.8 mg), and based on this observation it is concluded that the use as recommended of these 14 supplements resulting in estimated daily sibutramine dose levels from 4 to 36 mg day^{-1} might induce side effects. Furthermore, the use of six herbal supplements containing DMS (XXIII) or DDMS (VI-VIII, XIV, and XV) as recommended might induce side effects such as psychotic symptoms for DMS (Yuen et al. 2007; Chen et al. 2010) and cardiovascular effects for DDMS (Fil et al. 2011).

In conclusion, from the 50 herbal supplements sampled on the Dutch market, use of 20 supplements as recommended would result in estimated daily dose levels of sibutramine, DMS, DDMS, and/or phenolphthalein that could produce pharmacological effects. Furthermore, use of four out of ten herbal supplements as recommended would result in estimated daily phenolphthalein dose levels that would result in MOE values lower than 10,000. For short term-exposure (few weeks or months) MOE values may be two to three orders of magnitude higher.

To conclude, APIs should not be used as ingredients in food supplements. Such products should only be brought on the market as medicinal products because the legal framework for medicines is considerably better equipped to ensure the safe and effective use of these products. The current study shows that the use of food supplements with sibutramine, DMS, DDMS, and/or phenolphthalein could result in pharmacological effects.

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CHAPTER 6
General discussion

The aim of the present PhD thesis was to investigate the presence and actual levels of contaminants of concern in selected food supplements on the Dutch market and to estimate the associated health risks. Clay products for oral use, herbal food supplements claiming to enhance sexual potency and herbal food supplements used for weight loss were the food supplements studied. These three categories of food supplements were selected for the studies in the present thesis based on reports in literature on contaminations in food and feed.

After an introduction to the topic of interest (Chapter 1), the thesis continues with reports on the presence of metals, the metalloid arsenic and dioxins in clay products for oral use (Chapters 2 and 3). Clay products, known by a variety of names such as mabele, calabash chalk, sikor and pimba are traditionally consumed by pregnant women in Africa, Asia and Suriname, but also immigrants. Furthermore, clay is also used by non-pregnant women for certain health purposes in Western societies.

Chapter 2 focusses on the presence of metals and the metalloid arsenic in clay products. Aim of this study was to determine lead, arsenic, mercury and cadmium in clay products for oral use available on the Dutch market. Traditional clays originating from Africa (n=10) and Suriname (n=26), and health clays (n=27) were sampled from 2004 up to and including 2012. Total metal and metalloid contents were measured by ICP-MS and showed maximum levels of lead, arsenic, mercury and cadmium of 99.7, 45.1, 2.2 and 0.75 mg kg^{-1} respectively. The potential exposure was estimated from the determined concentration, the estimated daily use level of the clays and the estimated bioaccessibility of the different metals and arsenic. The intake estimates were compared to existing health based guidance values. For lead, the use of 34 of the 36 traditional clays and two of the 27 health clays would result in intake levels exceeding the toxicological limit by up to 20-fold. In the case of inorganic arsenic, the use of 15 of the 35 traditional clays and 11 of the 27 health clays would result in intake levels exceeding the toxicological limit for up to 19-fold. Although limited bioaccessibility from the clay may limit the exposure and exceedance of the health based guidance values, it was concluded that lead and arsenic intakes from some clay products could be of concern also because of their use by pregnant women and the potential developmental toxicity to the fetus.

In chapter 3 the contamination of clay products with dioxins was investigated. Certain types of clay are known to contain high concentrations of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and therefore the aim of this study was to determine the PCDD/F contents of orally consumed clays purchased on the Dutch market, also comparing them to levels in clays from African markets. From the Dutch market thirteen clay products were examined, including seven clay products of African and six of Suriname origin. In addition from seven African countries, twenty clay products were collected. All 33 clay products were screened with a cell-based bioassay

and those showing a high response were analyzed by gas chromatography/high resolution mass spectrometry (GC/HRMS). High PCDD/F concentrations were measured in three clay products from the Dutch market, ranging from 66 to 103 pg TEQ g^{-1}, whereas PCDD/F concentrations in the suspected clay products from African countries varied from 24 to 75 pg TEQ g^{-1}. In the WHO mother milk studies it was discovered that pooled human milk samples from some African countries contain relatively high dioxin levels. Therefore, congener patterns in African clay were compared with those of pooled human milk samples collected by WHO in eight African countries, to investigate a possible similarity of congener patterns from human milk with congener patterns from clay used for consumption. From the similarity between the patterns in clays and the human milk samples from the Democratic Republic of The Congo and Côte d'Ivoire, it can be concluded that there is probably a relationship with the consumption of contaminated clay. Frequent use of clay products during pregnancy may result in increased exposure of the mother and subsequently the developing fetus and new-born child. Based on the results of especially the presence of metals found in the traditional clay products, which were described in chapter 2, the Stichting Voedingscentrum Nederland -Netherlands Nutrition Centre Foundation-, started a campaign to discourage the use of pimba during pregnancy (Voedingscentrum 2009).

Chapters 4 and 5 report on the presence of active pharmacological ingredients (APIs) in herbal food supplements. Chapter 4 focussed on herbal food supplements, claiming to enhance sexual potency, which may contain deliberately added APIs that can be used for the treatment of erectile dysfunction. The aim of this study was to determine whether herbal food supplements on the Dutch market contain APIs known to inhibit phosphodiesterase type 5 (PDE-5), such as sildenafil and other known analogous PDE-5 inhibitors. Herbal food supplements intended to enhance sexual potency (n=71), and two soft drinks, were sampled from 2003 up to and including 2012. In 23 herbal supplements, nine different PDE-5 inhibitors were identified, in a few cases (n=3) more than one. The presence of these APIs was however not stated on the label. The amounts of PDE-5 inhibitors per dose unit were determined. Subsequently, the possible occurrence of potential pharmacological effects upon intake of the supplements with the detected PDE-5 inhibitors was estimated using data from scientific and patent literature regarding i) *in vitro* PDE-5 inhibitor activity of the detected APIs, ii) reported effective doses of registered drugs with PDE-5 inhibitor activity, and iii) similarity to other structural analogues. It was concluded that 18 of the 23 herbal food supplements with PDE-5 inhibitors, when used as recommended, would have pharmacological effects due to the added APIs. Based on similar observations the U.S. Food and Drug Administration (FDA) provided consumer health information about what they called the 'Hidden risks of erectile dysfunction "treatments" sold online' (http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm048386.htm). These con-

sumer health information includes the warning that men looking online for 'dietary supplements' to treat erectile dysfunction (ED) or enhance their sexual performance should beware that these products may contain prescription drugs or other undisclosed ingredients that can be harmful (FDA 2009).

In chapter 5 another group of herbal food supplements, claiming to reduce weight were investigated. These herbal food supplements may contain deliberately added active pharmacological ingredients (APIs) that can be used for the treatment of overweight and obesity. The aim of this study was to determine whether herbal food supplements for weight loss on the Dutch market contain APIs with weight loss properties. Herbal food supplements intended for weight loss (n=50), were sampled from August 2004 up to and including May 2013. A HPLC-DAD-MS/MS method was used to screen for the presence of the APIs in herbal supplements. In 24 samples the APIs sibutramine, desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant, sildenafil, and/or the laxative phenolphthalein were identified 41 times. The presence of these APIs was however not stated on the label. The potential pharmacological effects of the detected APIs were estimated using data from reported effective doses of approved drugs. Use of 20 of the 24 herbal food supplements, may result in potential pharmacological effects. Furthermore, risk assessment of phenolphthalein, a suspected carcinogen and found to be present in ten supplements, based on the Margin of Exposure (MOE) approach resulted in MOE values of 96-30,000. MOE values lower than 10,000 (96-220) were calculated for the daily intake levels of four out of these ten supplements in which phenolphthalein was found. However, taking into account that weight loss preparations may be used for only a few weeks or months rather than during lifetime, MOE values may be two to three orders of magnitude higher. The current study shows that the use of food supplements with sibutramine, DMS, DDMS, and/or phenolphthalein could result in both pharmacological but also other health effects.

Future perspectives

Given the results of the studies presented in this thesis, several topics for future actions and research priorities can be defined, and in the following sections several of these are discussed in more detail.

Health risks of food supplements and legislation

Already in 2004, EFSA pointed out that the use of botanicals, which include herbal supplements, is increasing (EFSA 2009[a]). Concerns related to the safety of herbal supplements containing botanicals or botanical preparations increase in significance as well. Since then, EFSA has issued documents on the safety of herbal food supplements such

as the 'Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements' (EFSA 2009[a]) and the 'Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements' (EFSA 2012[a]). However, documents mainly focus on the naturally occurring endogenous botanical toxins. However, from the thesis of Martena (2010) and from the results presented in this thesis it is concluded that also the presence of exogenous contaminants in food supplements such as metals and the metalloid arsenic, PCDD/Fs, and APIs may raise concern. In European regulatory framework regarding contaminants in foods, maximum levels are also set for metals lead, mercury and cadmium in food supplements (EC 2006).

To enforce actions of food supplements containing APIs, a different approach applies than to the products containing heavy metals, metalloids or PCDD/Fs. For these botanical products containing APIs it has to be investigated whether the API found is present in a pharmacologically relevant dose. A botanical product can then be classified as a medicinal product as defined in the medicinal products legislation. It is important to note that these pharmacologically relevant doses may or may not represent the dose of the API that can result in adverse effects. For a drug to be approved, the therapeutic effects need to outweigh the adverse effects. For a food supplement with APIs such an assessment is not made. For the quantification of the possible adverse health effects of non-registered APIs found in the herbal supplements, data are often lacking. In addition, the applicability of the Novel Food legislation (EC 1997) could be considered for evaluation of herbal supplements containing APIs. Novel foods are foods that have not been used for human consumption to a significant degree in the EU before 15 May 1997. Furthermore, novel foods can consist of or can be isolated from plants, but may not present a danger to the consumer or mislead the consumer. Therefore, prior to marketing a novel food, the company must apply to an EU member state competent authority for authorization, presenting the scientific information and a safety assessment report.

Selection of health based guidance values for risk assessment of metals and the metalloid arsenic found in clay products

In chapter 2 the safety of the contaminated clay products was assessed using health based guidance values which were set by risk assessment agencies such as RIVM, EFSA and JECFA (Baars et al. 2001; EC SCF 2001; 2003; IPCS 2000; 2003; JECFA 1982; 1983; 2007; 2011[a]; 2011[b]; EFSA 2008; 2009[b]; 2009[c]; 2010[a]; 2010[b]; 2012[b]; WHO 1996). These health based guidance values are in general based on data from toxicological studies in laboratory animals. In our study we applied the health based guidance values found in literature for the metals aluminum, cadmium, chromium, copper, iron, lead, mercury, zinc, and the metalloid arsenic. For our risk assessment in clay products we selected the

health based guidance values on the basis of chemical speciation of the metals and the metalloid in the clay matrix. Another criterion for selection was that the health based guidance value should be recently set. Furthermore, regarding the potential use during pregnancy, we selected, if present, the health based guidance value that was based on adverse effects on the fetus. However, upon finalization of our study for some elements such as aluminum (JECFA 2011[a]), arsenic (EFSA 2009[b]; JECFA 2011[b]), lead (EFSA 2010[b]), and inorganic mercury (JECFA 2011[b]; EFSA 2012[b]), new health based guidance values were set. These new health based guidance values were tolerable intake levels for adverse effects with a threshold such as for aluminum (JECFA 2011[a]) and inorganic mercury (JECFA 2011[b]; EFSA 2012[b]) or benchmark dose (BMD) values for adverse effects such as for arsenic (EFSA 2009[b]; JECFA 2011[b]), and lead (EFSA 2010[b]). The use of the BMD approach is advocated by EFSA for deriving a reference point for risk assessment. An advantage of the BMD approach is that all available dose-response data are used instead of only the NOAEL. Furthermore, the BMD approach takes the uncertainties in the dose-response data into account (EFSA 2009[d]). Table 1 shows to what extent lowering or increasing these health based guidance values or the use of alternative health based guidance values such as BMD values would result in a modification of the number of clay products exceeding the health based guidance values, considering the metals and the metalloid would be equally bioaccessible as the salts used in the critical toxicological studies used to derive the health based guidance values (100% bioaccessible scenario). From this Table it can be concluded that, based on the newly identified health based guidance values for aluminium and mercury the number of exceedances is not affected to a large extent.

Table 1. Number of clay products for which the use would lead to estimated intakes exceeding selected health based guidance values[a], assuming 100% bioaccessibility.

Metal/ metalloid	Health based guidance value (mg week^{-1})[a]	Type of health based guidance value	Health based guidance value set by	No of clay products exceeding health based guidance value/No tested per metal or metalloid
Aluminium	60	PTWI[b];TWI[c]	JECFA (2007); EFSA (2008)	55/55
	120	**PTWI[b]**	**JECFA (2011[a])**	**53/55**
Arsenic	**0.42**	**TDI[d]**	**RIVM (Baars et al. 2001)**	**26/61**
	0.13 - 3.36	**$BMDL_{01}$[e]**	**EFSA (2009[b])**	**49/61**
	1.26 (range 0.84 – 2.94)	$BMDL_{0.5}$[f]	JECFA (2011[b])	12/61
Lead	**1.5**	**PTWI[b]**	**IPCS (2000)**	**36/62**
	0.23	**$BMDL_{01}$[e]**	**EFSA (2010[b])**	**51/62**
Mercury	0.84	TDI[d]; TDI[d]	ICPS (2003); RIVM (Baars et al. 2001)	0/32
	1.68	**PTWI[b]; TWI[d]**	**JECFA (2011[b]); EFSA (2012[b])**	**0/32**

Notes: Values in bold were the values used in the chapter 2 for the safety assessments.

[a] expressed on a weekly basis and calculated for a 60 kg adult

[b] Provisional Tolerable Weekly Intake

[c] Tolerable Weekly Intake

[d] Tolerable Daily Intake

[e] Benchmark Dose Lower Confidence Limit of 1% extra risk

[f] Benchmark Dose Lower Confidence Limit of 0.5% extra risk

Matrix effects and risk assessment

Another topic that needs further attention in future studies is the bioaccessibility of the metals and the metalloid from the clay matrix. Such data on this bioaccessibility from the clay products were not available but we assumed that metals and the metalloid present in clay products would not be fully available for intestinal absorption. We estimated per metal and the metalloid arsenic the bioaccessible fractions, and included these in the risk assessment. This estimate was based on data available in the literature from *in vitro* bioaccessibility studies in soil fractions (Abrahams 1997; Aufreiter et al. 1997; Schoof and Nielsen 1997; Smith et al. 2000; 2009; Barnett and Turner 2001; Abrahams et al. 2006; Juhasz et al. 2007; Ljung et al. 2007; Palumbo-Roe and Klinck 2007; Madrid et al. 2008; Lamb et al. 2009; Morman et al. 2009; Zagury et al. 2009; Meunier et al. 2010; Roussel et al. 2010; Appleton et al. 2012). Further refinement of the risk assessment requires more detailed quantification of the bioaccesibility from the clay products actually investigated. Since this correction on bioaccesibility may significantly affect the exposure estimates (correction may be up to a reduction by more than 99% of the bioaccessibility for aluminum, iron and mercury), exact knowledge on bioaccesibility from the adequate food matrix will reduce the uncertainty in the risk assessment. Therefore, it is important to investigate this bioaccessibility in relation to the salts used in the toxicological studies used to derive the health based guidance values. The issue of how to deal with matrix effects in risk assessment is an important topic for future research on the use of clays.

Furthermore, possible nutrient interactions with the contaminants found in the food supplements exist and might affect the degree of toxicity of contaminants present in food supplements as well. Grapefruit juice for example, is known as a selective CYP3A4 inhibitor, and can increase the bioavailability of sildenafil (Jetter et al. 2002; Mason 2010), but the medicinal herb St. John's Wort induces the activity of CYP3A4 and is reported to decrease the bioavailability of sildenafil (Nowack 2008). Therefore, in order to refine the risk assessment, also nutrient interactions should be taken into consideration.

The results from the present study show that potential interactions of the food supplement matrix affect the risk assessment of contaminants present in food supplements. To adequately integrate this matrix or combination effect in the risk assessment is an important challenge for the future. EFSA has indicated that the matrix effects should be taken into account on a case-by-case basis (EFSA 2009[a]), and harmonisation on methods and procedures required to do so would be an important topic for future research.

Groups in the population of special concern

In the present thesis the focus was on food supplements used by groups of the population of special concern such as pregnant women and their unborn children (Chapters 2 and 3), and consumers suffering from erectile dysfunction (Chapter 4), overweight and obesity (Chapter 5). These consumers may be more vulnerable than 'healthy consumers' for the adverse effects resulting from the use of food supplements in which contaminants are found. In the following paragraphs these possible adverse effects resulting from consumption of the food supplements on each group of concern are discussed in some more detail.

The placenta is an important organ in the connection between mother and fetus. By this organ all necessary nutrients are delivered to the fetus, but the placenta also acts as a barrier for toxic substances including metals (Singh et al. 2010). However, a number of toxicants including metals are known to cross the placenta and can accumulate in the fetal tissues (Gundacker and Hengstschläger 2012). Critical periods or critical windows have been identified in the fetus development during which exposure to metals and the metalloid can be more harmful to the fetus because they can have adverse effects on the development of an organ or organ system such as the nervous system as is the case for lead (Baars et al. 2001). The nervous system mainly develops in the first trimester of pregnancy. In this early pregnancy it is reported that clay products are used by pregnant women to prevent morning sickness (De Korte 2006). Also PCDD/Fs have critical effects on the developing fetus (Van den Berg et al. 2000). Furthermore, lead and PCDD/Fs are known to accumulate in the skeleton (lead), liver and fat tissue (PCDD/Fs) (Gulson et al. 2003; Hoogenboom et al. 2010; Malish et al. 2010). During the lactation period these contaminants can be remobilized in the mother's body and transferred to the baby via the milk (EFSA 2010[b]; Malish et al. 2010) and therefore may pose an additional health risk for the baby.

In our study we found PDE-5 inhibitors present in herbal supplements for the enhancement of sexual potency. Studies show that decrease of sexual potency such as erectile dysfunction is associated with an increasing age or unhealthy lifestyle such as lack of exercise or smoking. Erectile dysfunction is associated with e.g. hypertension, hypercholesterolaemia, metabolic syndrome, and obesity (Derby et al. 2000; Hatzimouratidis et al. 2010; Gandaglia 2013). For the treatment of pulmonary hypertension, registered drugs such as Revatio® and Adcirca® are available on the market containing PDE-5 inhibitors as these are known to cause vasodilatation. Therefore, the unknown additional exposure to PDE-5 inhibitors through the use of herbal supplements for erectile dysfunction might negatively interfere with this type of treatment with registered drugs containing PDE-5 inhibitors, and patients might be at risk using such herbal supplements. Additionally, patients suffering from hypotension, who are not treated for this, might be at risk when using herbal supplements containing PDE-inhibitors.

The use of sibutramine is associated with cardiovascular symptoms such as an increased risk of serious, non-fatal cardiovascular events (stroke and heart attack), tachycardia, and (arterial) hypertension (Ioannides-Demos et al. 2006; Padwal & Majumdar 2007; Müller et al. 2009; Cohen & Ernst 2010; EMA 2010[a]). Furthermore, several psychiatric symptoms have been reported resulting from the use of sibutramine, desmethylsibutramine (DMS) and didesmethylsibutramine (DDMS) such as psychosis and (hypo)mania (Taflinski and Chojnacka 2000; Litvan and Alcoverro-Fortuny 2007; Yuen et al. 2007; Lee et al. 2008; Müller et al. 2009; Chen et al. 2010; Chong 2010; van Hunsel and van Grootheest 2011; Waszkiewicz et al. 2012). These examples illustrate that risk assessment of food supplements should pay attention to groups at extra risk especially when specific health conditions may trigger supplement use.

Risk-benefit balance

The results obtained in the present thesis showed levels of contaminants which can have adverse effects on the users and in case of clay products also on the offspring. However, in literature some possible beneficial effects resulting from the use of clay products are reported as well, such as reduction of morning sickness, reduction of acid reflux, supplementation of extra minerals, prevention of miscarriage and promotion of delivery (Danford 1982; Abrahams and Parsons 1996; Aufreiter et al. 1997; Geissler et al. 1999; Henry and Matthews Kwong 2003; De Korte 2006; Kawai et al. 2009). These claimed beneficial health effects, some of which might be placebo effects on the mother, might be beneficial for the health of the fetus as well. Furthermore, beneficial health effects, resulting from the high content of antioxidants such as flavonoids reported to be present in spices, herbs, supplements, and herb-containing medicaments may exist (Boots et al. 2008; Carlsen et al. 2010). In order to make a risk benefit analysis, both the adverse and beneficial effects should be properly quantified. The quantification of beneficial effects resulting from the use of clay products and herbal supplements is hampered by a lack of scientific data. Furthermore, different type of effects such as toxicological, infectious, psychological, and placebo effects need to be weighed and mutually compared. Therefore, these effects should be qualified in such a way that between the effects a comparison can be made. This would enable a risk-benefit assessment for food supplements weighing the possible health benefits against the risks (EFSA 2012[b]). This risk benefit balance for food supplements might be further explored in the (refinement) of risk assessment as well.

Contamination of herbal supplements with APIs and pharmacologial effectiveness of trace levels of APIs in herbal supplements

In the studies described in this thesis, APIs were found at levels of pharmacological relevance in herbal supplement for the enhancement of sexual potency and herbal supplements for weight loss (Chapter 4 and 5). This clearly indicates the deliberate addition of these APIs. However, also trace and low levels of APIs were found in some of the herbal supplements. Additionally, in some supplements more than one API was identified. A question can be raised about the possible source of this contamination of food supplements with APIs. The presence of these low levels or multiple APIs might be related to the quality of the starting material including these impurities. Furthermore, insufficient control of the production process such as inadequate cleaning between production runs might also result in these trace levels and a variety of APIs and API levels present in a herbal supplement.

In some of the herbal supplements used to enhance sexual potency and for weight loss trace levels of APIs were found. Based on available data, these trace levels on itself are not expected to be pharmacologically relevant. However, aside of the quality problem associated with manufacturing deficiencies which is discussed above, it is of importance to note that trace levels of APIs, may be in combination with other APIs present at higher levels, might add to the pharmacological effect of the supplement. This possible additive pharmacological effect of combinations of different APIs remains to be investigated. Due to the lack of data on dose-response curves, it was not possible to assess to which extent such low levels or the combination of compounds might still have a pharmacological effect, at least in some people, and could therefore have been intentionally added.

Drop outs from drug development

In chapters 4 and 5 the presence of APIs added to herbal supplements are reported. The analogous PDE-5 inhibitors found in herbal supplements, described in chapter 4, are often by-products of the typical drug development process (RIVM 2010; Venhuis and de Kaste 2012). This is an issue that may require more attention from a food safety perspective than presently realised. Drugs developed by pharmaceutical companies are subject to a scientific pre-market evaluation by health authorities such as EMA (Europe) and FDA (US) and these drugs can only enter the market after their efficicay and safety have been shown. However, in the process of drug development, large numbers of analogues may be developed that may for various reasons not enter the final stages of drug development and safety evaluation for market entrance. This implies that these drop outs from the drug development process do not undergo the full scientific safety evaluation because these APIs will not be a candidate for entering the market. This may

be for a variety of reasons, one of them being safety issues observed in early phases of drug development. As a result scientific data on pharmacological and toxicological features of these by-products are often lacking or not available to the public. Therefore, the intentional addition of these by-products to herbal food supplements, may result in unforseen adverse side effects in consumers.

Use of bioassays

Given that compounds of concern detected in food products on the market may be unknown analogues of drugs or other active compounds, it is of interest to consider increased use of bioassays for future screening purposes and quality control. Bioassays are able to detect chemicals with a certain related mode of action and thus facilitate the detection of unknown compounds with a certain mode of action. In our study we used such a cell-based bioassay for the screening of dioxin-like activity in clay products (Chapter 3). Application of this bioassay for screening of feed ingredients led to the discovery of various brominated flame retardants but also brominated dioxins in choline chloride, probably due to poor manufacturing conditions (Traag et al. 2009). Furthermore, in order to assess the pharmacological potency of PDE-5 inhibitors data from bioassays on the inhibition of PDE-5 activity were used (Chapter 4). Such a bioassay may also be applied for screening purposes. Moreover, various bioassays have been developed for estrogenic and androgenic substances, and have been used for screening of urine from calves and for animal feed (Bovee et al. 2006). Application of the yeast androgen assay led to the discovery of 1-methyltestosterone in supplements, which was overlooked by the analytical method (Rijk et al. 2009), and using the yeast estrogen assay, high levels of diethylstilbestrol (DES) were detected in a herbal product used for prostate problems and causing gynaecomastia in the patient (Toorians et al. 2010). For the screening of PDE-5 inhibitors, the use of a bioassay should be considered and can be based on the fact that PDE-5 inhibitors inhibit a single enzyme system such as the nitric oxide–cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 enzymes. For the screening of APIs in food supplements intended for weight loss, the use of a combination of bioassays should be considered as the APIs such as sibutramine, found in this type of food supplements, might act via different biological mechanisms. The use of bioassays for the screening of APIs in food supplements might prove to be useful for the detection of unknown compounds, since the currently used methods such as HPLC-DAD-MS/MS require that the compound is defined and known, which may not always be the case for APIs present in food supplements. Thus, bioassays can be used as rapid first screening and are to be followed by chemical analysis of suspected samples by more expensive chemical methods to identify and quantify the compounds causing the effects in the bioassay.

Safety of food supplements on the Dutch market

From this thesis the idea might arise that the Dutch market is dominated by unsafe food supplements. However, it is important to note that the sampling protocols, described in this thesis, were risk based and specifically targeted at finding food supplements that were more likely to be contaminated with metals, metalloids, dioxins and APIs. Therefore, these findings cannot be extrapolated to all food supplements present on the Dutch market. The type of retail channel is a factor influencing the chances on purchasing unsafe food supplements, with the retail via Internet and traditional shops being of particular concern. The Internet is a growing medium for the sales of food supplements with rapidly emerging and disappearing suppliers. Furthermore, the advertisements on Internet of these products often appear to lack information or to present disinformation, and consumers might not be able to assess the product information properly (Morris and Avorn 2003; Jordan and Haywood 2007; Vargas-Murga et al. 2011). This absence of information or information present in another language than the Dutch language might also apply to the products sold via traditional shops.

Conclusions

From our studies it can be concluded that, in addition to concerns over naturally occurring endogenous toxins present in herbal supplements, the presence of exogenous contaminants in herbal supplements can pose a health concern. The results of the present thesis also lead to the conclusion that in order to refine the risk assessment on the presence of contaminants such as metals, metalloids, dioxins and APIs in (herbal) food supplements more precise data are required on bioaccessibility of contaminants of concern from the food matrix. Also information on groups at increased risk may need to be increased. Additionally, the presence of APIs in herbal supplements, which may originate from drop outs in the drug development process, is of concern. In order to screen for unknown APIs the increased use of effect-based bioassays should be considered, as they have been shown to be successful in detecting unexpected and as yet unknown active ingredients. From the results obtained for the food supplements included in our study it can be concluded that the risk of adverse health effects might increase when food supplements are purchased via Internet or via traditional shops, and consumers should be aware that this presents a serious risk.

The overall conclusion from the work described in this thesis is that for food supplements ‘natural’ does not equal safe.

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Summary

Through the increasing use and availability of food supplements on the market, safety concerns relating to the safety of these food supplements are growing as well. The aim of the present PhD thesis was to investigate the presence and actual levels of contaminants of concern in selected food supplements on the Dutch market and to estimate the associated health risks.

First, in chapter 1, an overview is provided on the food supplements selected for the studies, which are clay products for oral use, herbal food supplements used to enhance sexual potency and herbal food supplements used for weight loss. Furthermore, an overview is given on the Dutch en European legal provisions for food supplements.

In the first study described in chapter 2 of this theses data are presented on the occurrence of metals such as lead, mercury and cadmium and the metalloid arsenic in clay products which are used via the oral route by pregnant and lactating women. For lead, the use of 34 of the 36 traditional clays and two of the 27 health clays would result in intake levels exceeding the selected health based guidance values, by up to 20-fold. In the case of inorganic arsenic, the use of 15 of the 35 traditional clays and 11 of the 27 health clays would result in intake levels exceeding the health based guidance values by up to 19-fold.

The second study, described in chapter 3 of the thesis, reports data on the presence of dioxins in 33 clay products, which were collected on the Dutch market and in some African countries. Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F) were detected in clay products from the Dutch market, in concentrations ranging from 66 to 103 pg TEQ g^{-1}, whereas PCDD/F concentrations in the suspected clay products from African countries varied from 24 to 75 pg TEQ g^{-1}. Furthermore, in this study congener patterns in African clay products were compared with those of pooled human milk samples collected by WHO in eight African countries, to investigate a possible relation between PCDD/Fs in human milk with contaminated clay used for consumption. From the similarity between the patterns in clays and the human milk samples from the Democratic Republic of The Congo and Côte d'Ivoire, it can be concluded that there is probably a relationship with the consumption of contaminated clay.

The aim of the third study, described in chapter 4 of this thesis, was to determine whether herbal food supplements on the Dutch market contain active pharmacological ingredients (APIs) known to inhibit phosphodiesterase type 5 (PDE-5), such as sildenafil and other known analogous PDE-5 inhibitors. Therefore, herbal food supplements in-

tended to enhance sexual potency (n=71), and two soft drinks, were analysed. In 23 herbal supplements, nine different PDE-5 inhibitors were identified, in a few cases (n=3) more than one. The presence of these APIs was however not stated on the label. Subsequently, it was estimated whether intake of the supplements with the detected PDE-5 inhibitors could result in pharmacological effects. It was concluded that 18 of the 23 herbal food supplements with PDE-5 inhibitors, when used as recommended, would have pharmacological effects due to the added APIs.

In the fourth study, described chapter 5 of in this thesis, another group of herbal food supplements, claiming to reduce weight, was investigated for the presence of APIs that can be used for the treatment of overweight and obesity. To this end, 30 herbal food supplements for weight loss on the Dutch market were collected and analysed for the presence of APIs with weight loss properties. In 24 samples the APIs sibutramine, desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant, sildenafil, and/or the laxative phenolphthalein were identified 41 times. The potential pharmacological effects of the detected APIs were estimated, and use of 20 of the 24 herbal food supplements, may result in potential pharmacological effects. Furthermore, a risk assessment of phenolphthalein regarding its carcinogenic effects, resulted in Margin of Exposure (MOE) values of 96-30,000. MOE values lower than the minimum required 10,000 (96-220) were calculated for the daily intake levels of four out of the ten supplements in which phenolphthalein was found. However, taking into account that weight loss preparations may be used for only a few weeks or months rather than during lifetime, MOE values may be two to three orders of magnitude higher. This study shows that the use of food supplements with sibutramine, DMS, DDMS, and/or phenolphthalein could result in both pharmacological but also other health effects.

From the studies described in this thesis it can be concluded (chapter 6) that, in addition to concerns over naturally occurring endogenous toxins present in herbal supplements, the presence of exogenous contaminants in herbal supplements can pose a health concern. Furthermore, the results of the present thesis also lead to the conclusion that in order to refine the risk assessment on the presence of contaminants such as metals, metalloids, dioxins and APIs in (herbal) food supplements more precise data are required on bioaccessibility of contaminants of concern from the food matrix. Also information on groups at increased risk may need to be increased. Additionally, the presence of APIs in herbal supplements, which may originate from drop outs in the drug development process, is of concern. In order to screen for unknown APIs the use of effect-based bioassays should be considered more often, as they have been shown to be successful in detecting unexpected and as yet unknown active ingredients. From the results obtained for the food supplements included in our studies it can be concluded that consumers should be aware that food supplements may not be without

risks. The overall conclusion from the work described in this thesis is that for food supplements ‘natural’ does not equal ‘safe’.

Samenvatting (Summary in Dutch)

Het toenemende gebruik en de ruime beschikbaarheid van voedingssupplementen op de markt geven reden tot zorg met betrekking tot de veiligheid van deze voedingssupplementen. Het doel van dit proefschrift is om de aanwezigheid en de gehalten van een aantal contaminanten in voedingssupplementen op de Nederlandse markt, die reden tot zorg voor de gezondheid geven, te onderzoeken en de daarmee samenhangende gezondheidsrisico's te beoordelen.

Allereerst wordt in hoofdstuk 1 een overzicht gegeven van de voedingssupplementen die zijn gekozen voor de studies. Dit zijn kleiproducten voor oraal gebruik, kruidensupplementen die gebruikt worden om de seksuele potentie te verhogen en kruidensupplementen die worden toegepast voor gewichtsverlies. Ook wordt in hoofdstuk 1 een overzicht gegeven van de huidige Nederlandse en Europese wetgeving voor voedingssupplementen.

In de eerste studie, die wordt beschreven in hoofdstuk 2 van dit proefschrift, worden gegevens gepresenteerd over het voorkomen van metalen zoals lood, kwik en cadmium en het metalloïde arseen in kleiproducten die worden geconsumeerd door zwangere en lacterende vrouwen. Voor lood zouden innameniveaus van 34 van de 36 traditionele kleiproducten en twee van de 27 gezondheidskleiproducten resulteren in innameniveaus die de geselecteerde gezondheidskundige grenswaarden tot wel 20 keer overschrijden. In het geval van anorganisch arseen zou het gebruik van 15 van de 35 traditionele kleiproducten en 11 van de 27 gezondsheidskleiproducten resulteren in innameniveaus die de gezondheidskundige grenswaarden tot wel 19 keer kunnen overschrijden.

In de tweede studie, beschreven in hoofdstuk 3, worden data gerapporteerd over de aanwezigheid van dioxinen in 33 kleiproducten die werden verzameld op de Nederlandse markt en in enkele Afrikaanse landen. Polychloordibenzo-p-dioxines en polychloordibenzofuranen (PCDD/F) werden gevonden in kleiproducten op de Nederlandse markt in concentraties, variërend van 66 tot 103 pg TEQ g^{-1}, terwijl PCDD/F-concentraties in de verdachte kleiproducten afkomstig van Afrikaanse landen varieerden van 24 tot 75 pg TEQ g^{-1}. Om een mogelijk verband tussen dioxines in moedermelk en het gebruik van besmette kleiproducten te onderzoeken, werden in deze studie de congenerpatronen van de kleiproducten vergeleken met die van gepoolde moedermelkmonsters die werden verzameld door de WHO in acht Afrikaanse landen. Uit de overeenkomst tussen de patronen in klei- en moedermelkmonsters afkomstig van de

Democratische Republiek Kongo en Ivoorkust, zou kunnen worden geconcludeerd dat er een mogelijke relatie is met de consumptie van gecontamineerde klei.

Het doel van de derde studie, beschreven in hoofdstuk 4, was om te bepalen of kruidensupplementen op de Nederlandse markt actieve farmacologische ingrediënten (API's) bevatten waarvan bekend is dat deze fosfodiesterase type 5 (PDE-5) remmen zoals sildenafil en andere bekende analoge PDE-5-remmers. Daarom werden kruidensupplementen (n=71) en twee frisdranken, bedoeld om de seksuele potentie te verhogen, geanalyseerd. In 23 kruidensupplementen werden negen verschillende PDE-5-remmers geïdentificeerd, in enkele gevallen (n=3) meer dan één. De aanwezigheid van deze API's was echter niet vermeld op het etiket. Vervolgens werden de mogelijke farmacologische effecten bij gebruik van deze supplementen met de aanwezige PDE-5 remmers geschat. Hieruit kon worden geconcludeerd dat 18 van de 23 kruidensupplementen met PDE-5 remmers, indien gebruikt als aanbevolen, zouden kunnen resulteren in farmacologische effecten als gevolg van de toegevoegde API's.

In de vierde studie, beschreven in hoofdstuk 5 van dit proefschrift, werd een andere groep van kruidensupplementen, bestemd voor gewichtsverlies, onderzocht op de aanwezigheid van API's die gebruikt kunnen worden voor behandeling van overgewicht en obesitas. Hiervoor werden 30 kruidensupplementen voor gewichtsverlies verzameld op de Nederlandse markt en geanalyseerd op de aanwezigheid van API's die gewichtsverlies kunnen veroorzaken. In 24 monsters werden de API's sibutramine, desmethylsibutramine (DMS), didesmethylsibutramine (DDMS), rimonabant, sildenafil, en/of de laxantia fenolfthaleïne 41 keer geïdentificeerd. Vervolgens werden de potentiële farmacologische effecten van de gedetecteerde gehaltes aan API's geschat en hieruit bleek dat het gebruik van 20 van de 24 kruidensupplementen zou kunnen resulteren in potentiële farmacologische effecten. Een risicobeoordeling van fenolfthaleïne, dat naast een laxerende werking ook carcinogene eigenschappen bezit, resulteerde in Margin of Exposure (MOE)-waarden van 96-30.000. MOE-waarden kleiner dan de gewenste 10.000 (96-220) werden berekend voor de dagelijkse innameniveaus van vier van de tien supplementen waarin fenolfthaleïne was gevonden. Echter, wanneer in aanmerking wordt genomen dat preparaten voor gewichtsverlies mogelijk gebruikt worden voor enkele weken of maanden in plaats van gedurende het gehele leven, dan zouden MOE-waarden twee of drie ordes van grootte hoger kunnen zijn. Deze studie laat zien dat het gebruik van supplementen met sibutramine, DMS, DDMS, en/of fenolfthaleïne kan resulteren in zowel farmacologische als ook andere gezondheidseffecten.

Op basis van de studies die in dit proefschrift zijn beschreven, kan worden geconcludeerd (hoofdstuk 6) dat, in aanvulling op de reden tot zorg met betrekking tot natuurlijk voorkomende endogene toxinen aanwezig in kruidensupplementen, ook de aanwezigheid van exogene contaminanten een reden tot zorg kunnen geven. Verder kan uit de resultaten van dit proefschrift ook worden geconcludeerd dat, om de risico-

beoordeling met betrekking tot de aanwezigheid van contaminanten zoals metalen, metalloïden, dioxines en API's in (kruiden)supplementen te verfijnen, meer exacte gegevens nodig zijn over de biobeschikbaarheid van deze contaminanten vanuit de voedingsmatrix. Ook zou meer informatie over gebruikersgroepen met een verhoogd risico beschikbaar moeten komen. Verder is de aanwezigheid van API's in kruidensupplementen, die mogelijk afkomstig zijn van drop outs van het ontwikkelingsproces van medicijnen, een zorg, aangezien deze vaak niet of nauwelijks zijn onderzocht op schadelijke effecten. Om te screenen op onbekende API's zou het toenemend gebruik van op effect gebaseerde bioassays moeten worden overwogen. Dit bleek in andere studies succesvol bij het detecteren van niet verwachte en onbekende actieve ingrediënten. Uit de resultaten die zijn verkregen over de voedingssupplementen uit onze studies, kan worden geconcludeerd dat consumenten zich bewust moeten zijn dat het gebruik van voedingssupplementen niet zonder risico's is. De algemene conclusie van het werk beschreven in dit proefschrift is dat voor voedingssupplementen 'natuurlijk' niet automatisch 'veilig' betekent.

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Curriculum Vitae

Noortje Maria Reeuwijk was born on 30th of October 1968 in Lisse, the Netherlands. In 1999 she obtained her DVM degree at the Veterinary Faculty of Utrecht University, the Netherlands, specializing in large animal medicine and veterinary public health. After obtaining her DVM degree, she worked in 2000 and 2001 as a poultry veterinarian in a private practice. Mid 2001, she took a position at the Netherlands Food and Consumer Product Safety Authority (NVWA) as a food inspector. Until 2007, she predominantly conducted official controls in food industry. Furthermore, she carried out investigations on zoonotic infections on a case by case basis. Based on the results of these investigations, in 2003 she started a residency in Veterinary Public Health under the supervision of prof. Dr. F. van Knapen within the Interfaculty Institute for Risk Assessment Sciences (IRAS) of Utrecht University. In 2009 she became a diplomate in Veterinary Public Health. From 2007 to 2011 she worked on the development of enforcement strategies in food industry, and became involved in the enforcement of the safety of food supplements. As a result, she started a PhD course in 2010 under supervision of prof. dr. ir. I.M.C.M. Rietjens of the Department of Toxicology of Wageningen University, the Netherlands, combined with her position at the NVWA. In addition, in 2012 she graduated from the Postgraduate Education in Toxicology course, which is offered by a cooperation of eight Dutch Universities.

Currently, she is working at the Office for Risk Assessment and Research (BuRO) of the NVWA.

List of publications

Published manuscripts

Reeuwijk NM, Venhuis BJ, de Kaste D, Hoogenboom LAP, Rietjens IMCM, Martena MJ. 2013. Sildenafil and analogous phosphodiesterase type 5 (PDE-5) inhibitors in herbal food supple-ments sampled on the Dutch market. Food Addit Contam A. 30(12):2027–2034.

Reeuwijk NM, Klerx WNM, Kooijman M, Hoogenboom LAP, Rietjens IMCM, Martena MJ. 2013. Levels of lead, arsenic, mercury and cadmium in clays for oral use on the Dutch market and estimation of associated risks. Food Addit Contam A. 30(9):1535-1545.

Reeuwijk NM, Talidda A, Malisch R, Kotz A, Tritscher A, Fiedler H, Zeilmaker MJ, Kooijman M, Wienk KJH, Traag WA, Hoogenboom LAP. 2013. Dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzo-furans) in traditional clay products used during pregnancy. Chemosphere. 90:1678–1685.

Published manuscripts non peer-reviewed

Reeuwijk N, van Dijk G, de Groot H, van Knapen F. 2009. Risico op een infectie met Salmonella bij mensen door het houden van reptielen/Risk of human Salmonella infection by keeping snakes. Litteratura Serpentium 2009, jaargang/volume 29, nummer/No. 4, 148-153, European Snake Society.

Reeuwijk N, van Dijk G, de Groot H, van Knapen F. 2009. Een infectie met Salmonella door het houden van reptielen. Dier-en-Arts, nummer 11, 2009, 394-399. Overname artikel uit Infectieziekten Bulletin.

Reeuwijk N, de Groot H, Klöpping E, van Knapen F. 2009. Begraven van (gezelschaps)dieren; wetgeving, mogelijke risico's en aanbevelingen. Tijdschrift voor Diergeneeskunde, Deel 134, Aflevering 14-15, 15 juli/1 augustus 2009, 616-620.

Reeuwijk NM, van Dijk G, Moïze de Chateleux WSJM, de Groot HN, van Knapen F. 2009. Een infectie met Salmonella door het houden van reptielen. Infectieziekten Bulletin, RIVM, jaar-gang 2, nummer 3, april 2009, 88-91.

Reeuwijk NM, de Groot HN. 2008. Mogelijke gezondheidsrisico's door het consumeren van rauwe paardenmelk. Infectieziekten Bulletin, RIVM, jaargang 19, nummer 7, september 2008, 223-225.

Manuscripts submitted

Reeuwijk NM, Venhuis BJ, de Kaste D, Hoogenboom LAP, Rietjens IMCM, Martena MJ. Active pharmaceutical ingredients detected in herbal food supplements for weight loss sampled on the Dutch market [submitted for publication].

Overview of completed training activities

Discipline specific training activities

Courses

PET: cell toxicology	2011
PET: molecular toxicology	2011
PET: reproductive toxicology	2011
PET: eco toxicology	2011
PET: medical and forensic toxicology	2011
PET: mutagenesis and carcinogenesis	2012

Meetings

NVT: annual meeting: current developments in non-clinical models for the safety assessment of pharmaceuticals	2011
Fresenius Conference: food safety and dietary risk assessment	2012
NVT section risk assessment meeting: threshold of toxicological concern	2013

General courses

Boertien Groep: time management	2007-2008
VU/EMGO: epidemiological research; set up and interpretation (A0101)	2008
MU-LC: advanced writing for sciences	2009
PET: toxicological risk assessment	2011
PET: risk communication	2012
BTSF-EC: chemical risk assessment in food	2012

Optionals

Preparation of research proposal	2009
FVE: ad hoc working group on hygiene (member)	2006-2009
VMT: contact group food safety and food technology (member)	2007-2011
Product Board Arable Products: expert group RiskPlaza (member)	2009-2010

Abbreviations

BTSF-EC:	Better Training for Safer Food - European Commission
FVE:	Federation of Veterinarians of Europe
MU-LC:	Maastricht University - Language Centre
NVT:	Netherlands Society of Toxicology
RiskPlaza:	database of information about the food safety of ingredients
PET:	Postgraduate Education in Toxicology
VMT:	magazine for food industry
VU/EMGO:	VU University Medical Center Amsterdam/EMGO Institute for Health Care Research

Disclaimer

The views expressed in this thesis are those of the author and co-authors and do not reflect the official policy or position of the NVWA, and/or any scientific institute and/or any Ministry of the Netherlands.

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